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METHODOLOGICAL STUDIES ON THE DETERMINATION OF RESPIRATION AND ETHYLENE PRODUCTION OF FRUITS AND VEGETABLES

E. KOVÁCS and K. VAS

(Received May 8, 1972)

Methodological studies were carried out to determine the respiration and ethylene production of fruits and vegetables.

Oxygen consumption was measured by a modified version of the Warburg method. Changes in gas volume taking place during a given time period were related to unit (a) weight, (b) surface or (c) volume of the fruit.

Standard deviation was lowest with the values calculated for unit weight. Values calculated for unit volume scarcely differed from the former, but the accurate determination of volume is difficult.

Carbon dioxide and ethylene production were determined by gas chromatography. Both gases were determined simultaneously, using a heat conductivity detector for carbon dioxide and a flame ionization detector for ethylene.

Air samples containing the carbon dioxide and the ethylene are transported by the carrier gas (helium) and a distributor to the two types of detector.

The method is rapid and its range can be extended between wide limits by changing the sensitivity of the detectors.

To follow up the ripening processes of fruits and vegetables is important, because apart from the quality of the raw material, the efficiency of storage also depends on the stage of ripeness.

The knowledge of respiration and ethylene production of sound fruits and vegetables is important from the point of view of storage physiology.

Respiration can be measured by colorimetric determination of CO_2 formation or O_2 consumption (CLAYPOOL & KEEFER, 1942), by titrimetry (BIALE *et al.*, 1954; MEIGH *et al.*, 1967), by spectrophotometry (GREEN *et al.*, 1969), by gas chromatography (LUH & CHAUDRY, 1961; MAXIE *et al.*, 1966; MEIGH & FILMER, 1969), or by an oxygen analyzer, on the basis of the paramagnetic properties of oxygen (LEE *et al.*, 1968; ROBINSON & COOPER, 1970).

Ethylene is either determined chemically by volumetric analysis (YOUNG *et al.*, 1952) or by gas chromatography (MEIGH, 1960; MEIGH *et al.*, 1967, 1969; LEE *et al.*, 1968; etc.)

The aim of the present studies was to elaborate a rapid, accurate method facilitating the parallel study of a sufficient number of samples and the statistical evaluation of the results.

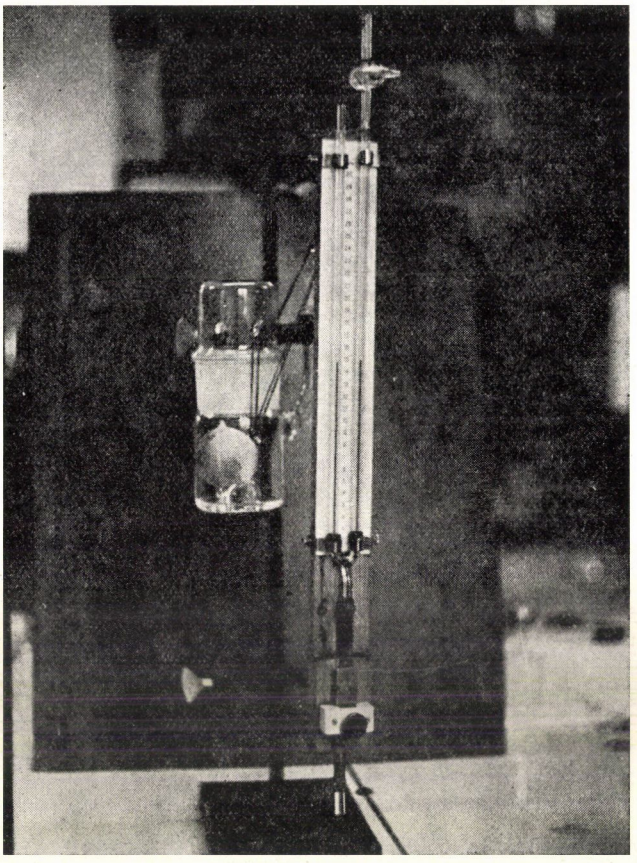

Fig. 1. Modified Warburg apparatus for measurement of oxygen consumption

1. Materials and methods

1.1. Determination of oxygen consumption by the modified Warburg method

1.1.1. Description of the apparatus. A Warburg-type manometer was used, but the traditional reaction flask was replaced by a container suitable to hold whole fruits (Fig. 1).

The volume of the air space of the flask was kept at the minimum (700—750 ml) to reduce the time of measurement as far as possible.

For the period of measurement the flasks were placed in a water bath, to maintain a constant temperature of air and raw material.

The carbon dioxide produced by the raw material was collected by absorption in 20% KOH, and oxygen consumption was determined on the basis of pressure change.

Pressure reduction taking place in a predetermined time period (20 or 30 minutes) was expressed as change in gas volume and this was calculated for unit weight of fruit and unit time, to express respiration intensity in terms of oxygen ml · h^{-1} · kg^{-1} raw fruit weight.

Apart from unit weight, the results were also calculated for unit volume (cm^3) and unit surface (cm^2).

Respiration intensity (L) per unit weight is calculated by the formula

$$L = \frac{h \cdot k \cdot 60 \cdot 1\,000}{t \cdot s} \; \mu l \cdot h^{-1} \cdot kg^{-1} .$$

Respiration intensity per unit volume is

$$L = \frac{h \cdot K \cdot 60}{6 \cdot k} \; \mu l \cdot h^{-1} \cdot cm^{-3} .$$

K (volume of the fruit) is expressed in cm^3, where

$$K = \frac{4}{3} \pi R r^2 .$$

Respiration intensity for unit surface area is

$$L = \frac{h \cdot k \cdot 60}{t \cdot A_f} \; \mu l \cdot h^{-1} \cdot cm^{-2},$$

if A_f (surface of fruit) is expressed in cm^2, where

$$A_f = 2\pi r^2 + \frac{2\pi R^2 r}{\sqrt{R^2 - r^2}} \arccos \frac{r}{R} \qquad R > r .$$

Symbols used:

h = change of manometric record, mmHg
k = flask constant, μl
t = time of measurement, minutes
s = weight of the samples examined, g
K = volume of fruit, presuming a rotational ellipsoid, cm^3
R = longer radius, cm
r = shorter radius, cm
A_f = surface area, presuming a rotational ellipsoid, cm^2

The data were evaluated in a *Razdan 3* computer, on the basis of the method described by UMBREIT *et al.* (1964).

1.1.2. Experimental procedure. Prior to the experiment, the samples were incubated at the experimental temperature in polyethylene pouches in a water bath.

25 ml of 20% KOH was pipetted into each reaction flask, the sample was added and the flasks were closed for further incubation in the arrangement shown in Fig. 1. Incubation was continued until the manometer did not show further pressure change. Then the manometers were closed and values of pressure changes taking place over a given time (20—30 minutes) were recorded and calculations made as described before.

1.2. Determination of carbon dioxide and ethylene

1.2.1. Apparatus. A two-column 5750 Hewlett-Packard gas chromatograph was used.

Parameters:

Detectors: flame ionization detector; heat conductivity detector
Column: stainless steel tube of 152.4 cm length with an inner diameter of 0.5 cm
Column pack: *Porapak R* (80—100 mesh)
Carrier gas: helium
Carrier gas velocity: 30 ml · min^{-1}, gas pressure at exit: 5 atm
Column temperature: 50—100°C, at 10°C · min^{-1} temperature programme
Injection port: 75°C
Temperature of the flame ionization detector: 140°C
Flow rate of hydrogen: 25 ml · min^{-1}
Air flow rate: 500 ml · min^{-1}
Temperature of the heat conductivity detector: 90°C
Bridge voltage: 130 mA

1.2.2. Quantity of samples measured. The concentrations of ethylene and carbon dioxide were 1 ml/1 000 ml air and 1 ml/100 ml air, respectively. Samples of 0.1 — 5 ml thus contained 0.1 μl — 5 μl ethylene or 1 — 50 μl carbon dioxide.

The samples of air containing ethylene and carbon dioxide, resp., were injected into the apparatus by means of a Hamilton 10-ml or 5-ml gas pipette and the distributor connected to the end of the column directed a part of the sample to the flame ionization detector, the other part to the heat conductivity detector.

The results of detector measurements were evaluated quantitatively.

2. Results

2.1. Determination of oxygen

Fresh apricots served as test material. Respiration of 8—20 individual fruits was measured and averages were calculated as shown in Table 1.

Table 1

Respiration intensity of apricots under various conditions of storage
(Storage temperatures are given in the Table; measurements were carried out at 25 ± 0.3°C)

Conditions of storage	Time of measurement (days)	N	Oxygen consumption								
			$ml \cdot h^{-1} \cdot kg^{-1}$			$ml \cdot h^{-1} \cdot cm^{-2}$			$ml \cdot h^{-1} \cdot cm^{-3}$		
			$\bar{X}$	s	v	$\bar{X}$	s	v	$\bar{X}$	s	v
At room temperature	0	8	48.1088	4.2199	8.77	25.1824	3.7774	15.00	56.0682	6.6550	11.87
(19–21°C, 40–50% RH)	1	9	53.7586	7.3478	13.67	29.0241	8.6966	29.96	65.8534	11.1240	16.89
	2	9	60.7540	10.4309	17.17	32.5252	7.6522	23.50	70.7691	12.4804	17.60
In refrigerator	0	9	60.7359	10.7199	17.64	32.8035	6.2285	18.99	67.3076	13.5135	20.07
(2–6°C, 70–90% RH)	1	20	57.9712	18.5582	32.01	25.1516	7.5351	29.96	66.0665	20.2298	30.62
	2	10	49.9135	10.7300	21.49	22.6283	6.1336	27.10	58.9352	11.6997	19.85

N = number of parallel measurements
X = mean of respiration intensity data
s = standard deviation
v = coefficient of variation

Part of the apricots were stored at room temperature (19—21°C, 40—50% RH) and measured at 25°C, part were stored in the refrigerator (2—6°C, 70—90% RH) and also examined at 25°C.

Fruits are not usually stored at the temperature of measurement. Respiration intensities should, however, preferably be measured at the storage temperature.

That the temperature of the sample should be identical with the temperature of the measurement is a prerequisite of reproducibility. Warming of cold-stored produces to the temperature of measurement requires a long incubation process (PAIS *et al.*, 1970), during which the respiration intensity of the sample will as a rule change. In this laboratory, incubation time was reduced by placing the samples in polyethylene pouches in a water bath of appropriate temperature for 30 minutes prior to measurement.

As can be seen in Table 1, the coefficient of variation of respiration values per unit weight varied between 8 and 17% for fruits originally stored at room temperature, and between 17—32% for those stored in the cold. The greater coefficient of variation of the latter measurements may have been due to the difference in the temperature of the fruit.

Respiration intensity is usually related to unit weight. Since we performed measurements on individual fruits, it was possible to calculate relative values for other units (cm^3 and cm^2) as well. Values for unit volume and their deviation roughly corresponded with those for unit weight.

Respiration for unit surface area (cm^2) differed in absolute value from those obtained for the other two units.

2.2. Determination of carbon dioxide and ethylene

A calibration curve for carbon dioxide is shown in Fig. 2. The minimum measurable concentration of CO_2 was ca. 0.5 $\mu l \cdot ml^{-1}$.

Results of the calibration experiment with ethylene are shown in Fig. 3; the minimum measurable concentration of ethylene was 0.1 $\mu l \cdot l^{-1}$ air.

3. Conclusions

Respiration measurements on fruits and vegetables usually require several kilograms of raw material for 2 or 3 parallel measurements. In most cases the effects of various treatments are studied simultaneously, and treatment numbers are increased at the cost of replicates. Thus the results are not sufficient for statistical evaluation.

Our method is based on measurements on individual fruits or vegetables on the grounds that more information is likely to be obtained from individual, but numerous measurements than from a single measurement carried out on an equal total amount of produce.

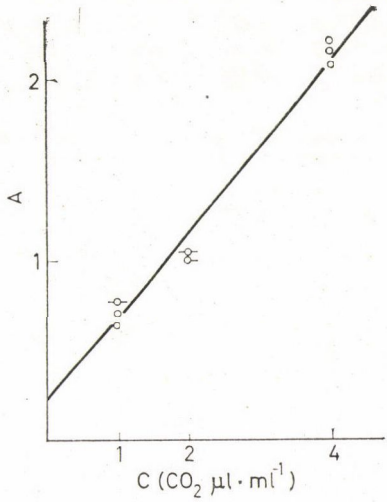

Fig. 2. Calibration curve for carbon dioxide. A: area below curve expressed in planimeter units; C: concentration; r: correlation coefficient = 0.9868; A: 0.2105 + 0.4768 · C; average coefficient of variation: 2.7%; sensitivity: 2

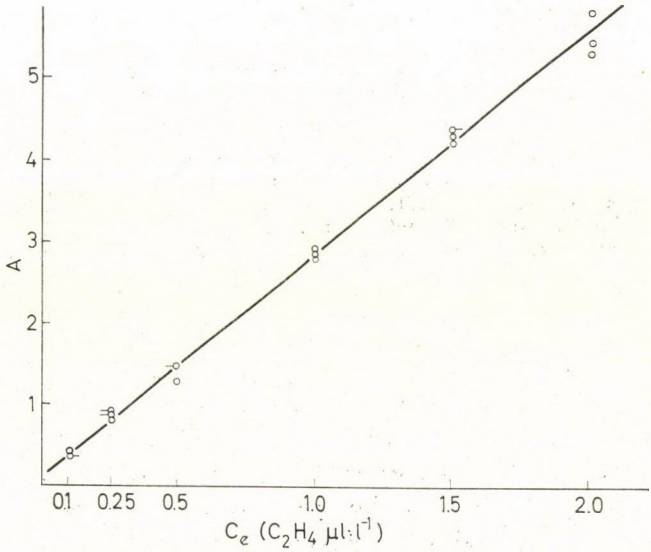

Fig. 3. Calibration curve for ethylene. A: area below curve expressed in planimeter units; r: correlation coefficient = 0.9981; C_e = concentration; A = 0.1449 + 2.7138 · C_e average coefficient of variation: 2.8%; sensitivity: 1.10^2

With this method a single person can perform 15—20 parallel measurements within an hour. The technique is simple and any temperature of measurement can be provided by appropriate adjustement of the water bath, independently of ambient temperature. Given a cooling thermostat low temperatures can also be applied.

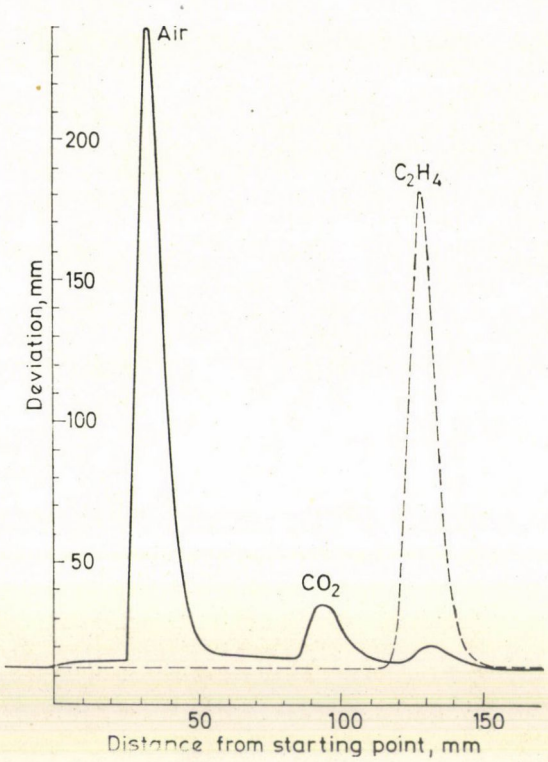

Fig. 4. Determination by gas chromatography of a mixture of carbon dioxide, ethylene and air. The curve represents the analysis of a 4-ml sample, under consideration of the following parameters: (156 ml air/1.5 ml ethylene) 6 ml CO_2, *Porapak R* column, sensitivity of the heat conductivity detector: 128, sensitivity of the flame ionization detector: 8.10^2, temperature range: 40—120°C, at a temperature programme of 10°C · min $^{-1}$

The procedure is simple and economical. It facilitates the performance of measurements at a temperature equal to the storage temperature, thus long incubation periods for adjusting the temperature of the sample can be eliminated (PAIS *et al.*, 1970). In our experiments, the lowest measurable ethylene concentration was 0.1 $\mu l \cdot l^{-1}$ while the lowest concentration measured by GALLIARD and GREY (1969) was 0.4 $\mu l \cdot l^{-1}$.

As to the reproducibility of the results, GALLIARD and GREY (1969) found the variation coefficient to be 2.26 %; in our hands it was almost identical:2.7 %.

Literature is only available on ethylene; simultaneous gas chromatographic determination of carbon dioxide and ethylene has not yet been reported.

The simultaneous gas chromatographic determination of the two gases by our method is illustrated graphically in Fig. 4.

We consider the main advantage of our method that the simultaneous measurement of the two gases greatly simplifies the technique, and at the same time yields a more accurate information on the test material than other methods.

*

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Thanks are due to Zs. VÖRÖS for the conscientious performance of the oxygen determinations.

One of us (E. K.) is indebted to the International Atomic Energy Agency for the fellowship which enabled the completion of part of this work in the FRG.

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EFFECT OF IONIZING RADIATIONS ON SOME ORGANOLEPTIC CHARACTERISTICS OF EDIBLE MUSHROOM

E. KOVÁCS and K. VAS

(Received May 8, 1972)

Organoleptic characteristics — colour, odour, flavour and texture — of edible mushrooms *(Agaricus bisporus)* in different stages of ripeness (half-ripe, ripe) were examined as a function of radiation dose, storage time as well as storage temperature and humidity (3—5°C, 70—90% RH; 16°C, 65—75% RH).

The results are summarized as follows:

— Immediately after irradiation the mushrooms assumed a slight brownish hue which, however, did not change on further storage. (The brown discoloration of untreated mushroom progressed in the course of storage.)

— The colour of the irradiated mushroom scarcely changed with storage time (6th, 11th or 13th day).

— Other examined properties (odour, flavour, texture) were not affected by irradiation either immediately upon treatment or during further storage.

— Ionizing radiation was found to exert favourable influence upon the organoleptic properties of picked mushrooms, and to delay the deterioration of these properties.

— Radiation treatment was found to preserve the characteristic mushroom odour and this is considered an advantage in canning.

The recent increase of public interest in up-to-date nutrition resulted in a rising demand for cultivated mushrooms all over the world. Mushrooms have become an indispensable raw material of modern diet. They contain a considerable amount of protein (4.9% on dry matter basis), carbohydrate (3.6%) and a low amount of fat (0.2%). Their nutritive value is increased by the fact that mushroom protein is an almost complete protein and 90% of it is metabolized (BESSENYEI, 1967; SOMOS, 1967).

The average vitamin content of mushroom is as follows:

	$mg \cdot kg^{-1}$
vitamin B_1	1.1
vitamin B_2	5.5
vitamin C	86.0

Mushrooms are known to be extremely perishable. Treatment with ionizing radiation as a new method for improving the keeping quality of mushrooms was first described by STADEN (1964; 1965a, b, c) and his good results were later confirmed by MACQUEEN *et al.* (1966), KOVÁCS *et al.* (1968a, b, 1970), STOLLER (1968) and GILL *et al.* (1969).

Radiation treatment to prevent senescence can only be recommended if it does not impair the organoleptic quality. Therefore, parallel to the study of the possibilities of physiological intervention it is desirable to carry out sensory evaluations.

1. Materials and methods

1.1. Test material

Raw material for examination was procured from the Mushroom Production Unit of the "Duna" Agricultural Cooperative, Budapest. Mushrooms *(Agaricus bisporus)* were studied at two stages of ripeness, as characterized in an earlier paper (KOVÁCS *et al.*, 1968a).

The examined mushrooms were divided into two groups, on the basis of cap diameter:

1) 3.1—4.0 cm,
2) 4.1—5.0 cm.

1.2. Irradiation

Irradiations were carried out in the ^{60}Co panoramic gamma radiation sources of 13 kCi and 80 kCi activity, respectively, Institute for Isotopes, of the Hungarian Academy of Sciences.

The dose rate varied between 1.2 and 30 krad $\cdot$ h^{-1} with the first irradiator and between 25 and 300 krad $\cdot$ h^{-1} with the second one.

In every case, storage conditions are described in the explanations to the figures, because results originate from different series of experiments.

1.3. Organoleptic evaluation

Chopped mushrooms of given weight were boiled for 20 minutes in a constant volume of water containing 2% NaCl and 0.2% citric acid. After cooking, organoleptic evaluation was performed by the same panel of 10 judges.

Colour, odour, flavour and texture were examined and evaluated by KRAMER's ranking system (1960). Samples were ranked according to scores, the sample receiving the highest score ranked 1. Rank sums were plotted as a function of radiation dose and storage time. Rank sums plotted in the figure represent limit values, dependent on the number of panelists and of samples. Values below or above the limit values stand for samples the sensory quality of which differ at 95 or 99% probability level from the rest of samples. Rank sums below the lower limit value belong to significantly or highly significantly better samples, while rank sums above the upper limit value are significantly or highly significantly worse than the rest of samples.

2. Results

Organoleptic evaluation carried out (KOVÁCS *et al.*, 1968a, b) immediately upon irradiation in earlier storage experiments showed that this treatment did not cause deterioration of the organoleptic quality of the mushrooms. As to

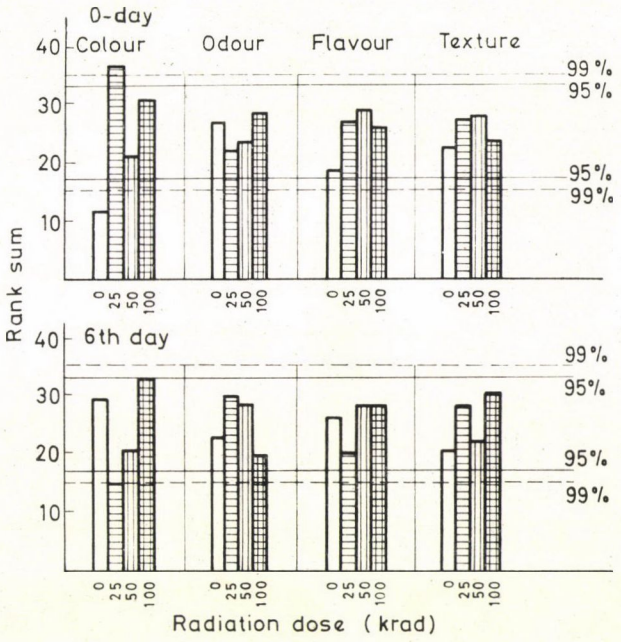

Fig. 1. Organoleptic evaluation of untreated and radiation-treated mushrooms, immediately after irradiation and after 6 days of cold storage (6—8°C, 65—66% RH)

colour, samples exposed to 25 krad had the deepest brown shade, the difference being significant at 99% probability level.

Examinations performed on the 6th day of storage revealed no significant differences between samples except for the one exposed to 25 krad, the colour of which was better, at the 99% probability level, than that of the rest (Fig. 1).

Results of the organoleptic evaluation of mushrooms untreated and treated with different radiation doses and subsequently stored in the cold for 13 days are shown in Fig. 2.

As can be seen, the characteristic mushroom colour is preserved with increasing radiation dose. The colour of samples treated with 100 krad was better than that of the rest at the 99% probability level.

The preservation of odour and flavour was unrelated to the applied dose of irradiation.

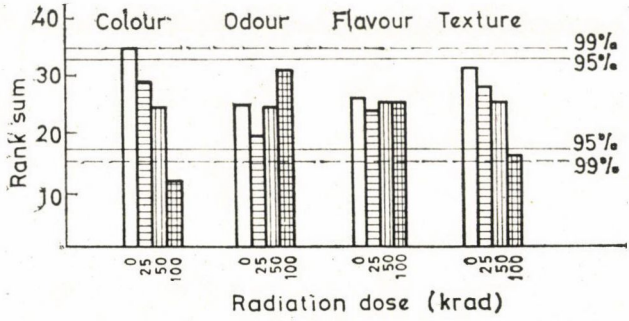

Fig. 2. Organoleptic evaluation of untreated and radiation-treated mushrooms after cold storage (6—8°C, 65—66% RH) of 13 days

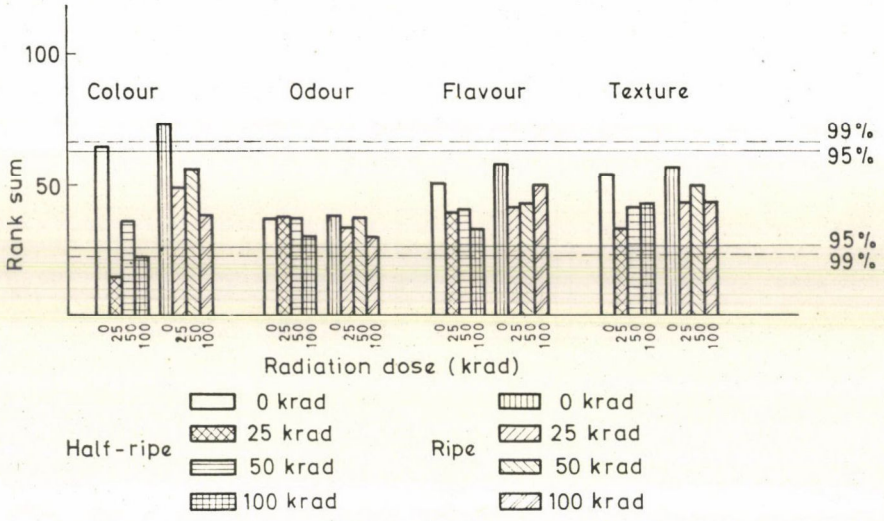

Fig. 3. Organoleptic evaluation of untreated and irradiated, freshly picked, half-ripe and ripe mushrooms after 9 days of storage (4—7°C, 67—90% RH)

The texture of the sample treated with 100 krad was significantly (95%) better in comparison to the rest.

Sensory properties as affected by radiation treatment in different stages of ripeness are shown in Fig. 3.

As can be seen, the colour of half-ripe mushrooms exposed to 25 and 100 krad was better, at the 99% probability level than that of the rest. Ripe mushrooms treated with various radiation doses did not differ from one another.

The colour of untreated mushrooms was significantly worse in comparison to the rest. Irradiation at different stages of ripeness produced no difference in respect of odour, flavour and texture.

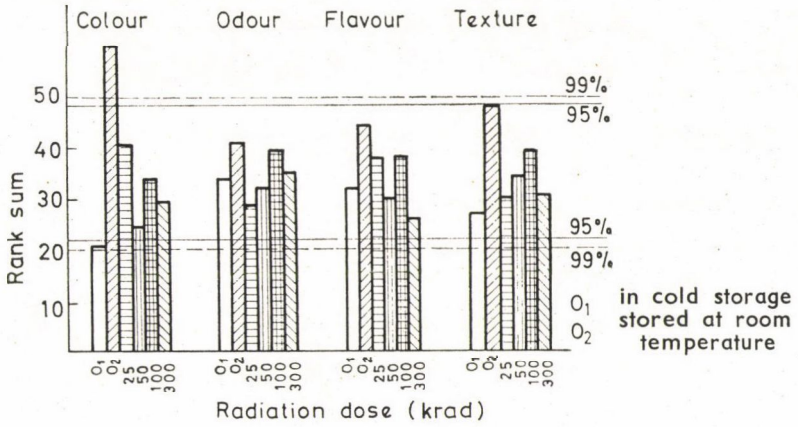

Fig. 4. Organoleptic quality of mushrooms irradiated after 2 days of cold storage and evaluated immediately after irradiation

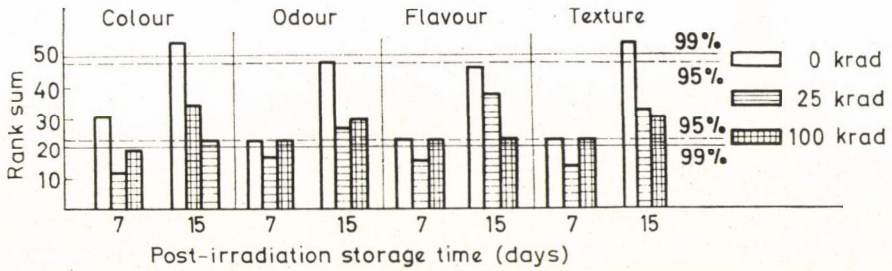

Fig. 5. Organoleptic quality of mushrooms irradiated after 2 days of cold storage (5—8°C, 70—90% RH) as a function of storage time after treatment

If mushrooms were irradiated only 2 days after harvest, their organoleptic properties changed as shown in Fig. 4, which was based on evaluation performed immediately after irradiation.

Results obtained after 7 and 15 days of storage can be seen in Fig. 5.

After storage for 7 days (5—8°C, 70—90% RH), samples treated with 25 krad were superior to the control in respect of colour, flavour and texture, those treated with 100 krad in respect of odour, flavour and texture.

After 15 days, the lot exposed to 25 krad had better colour, flavour and texture than the untreated control sample. The organoleptic evaluation of mushrooms stored at room temperature (16—18°C, 65% RH) for 11 days after irradiation is summarized in Fig. 6.

The colour of the untreated lot was inferior at 95% probability level, to that of the rest while the colour of the lot treated with 300 krad was significantly superior. Other properties did not generally differ after exposure to various radiation dose levels.

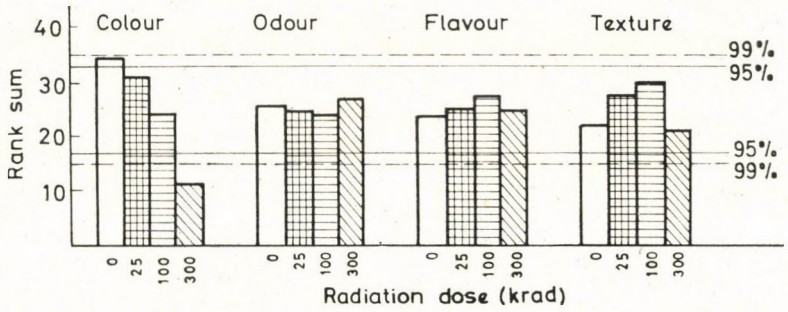

Fig. 6. Organoleptic quality of mushrooms irradiated immediately after harvest and stored at room temperature (16—18°C, 65% RH)

3. Conclusions

It follows from the results that radiation treatment had no immediate effect on the colour, odour, flavour and texture of mushrooms.

It is a general observation that irradiated mushrooms taste better than untreated ones (MERCIER & MACQUEEN, 1965; STADEN, 1966; KOVÁCS *et al.*, 1968a, b).

Mushrooms stored at 10°C after treatment with 500 krad were superior to untreated ones in respect of organoleptic quality.

Radiation treatment, storage temperature and stage of ripeness caused no difference in the odour of mushrooms. A distinct quality deterioration occurred in untreated controls.

The texture becomes tough with ageing of the tissues. Several authors reported that irradiation causes a temporary softening which, however, disappears during further storage.

In the present experiments, the texture of the mushrooms did not change either immediately after irradiation, or later. The probable reason is that already low radiation doses had a favourable influence on the physiological state, preventing the impairment of cell wall elements; the slowing down of life functions may also have contributed to the preservation of texture. GORMLEY (1969) found a close correlation between fresh weight, solids content and texture of mushrooms indicating the influence of the stage of ripeness on texture.

Radiation treatment is immediately followed by a slight brownish discoloration which, however, does not increase on further storage in contrast to the intensive browning of non-irradiated controls. The tendency of browning is observed mainly when irradiation is carried out with an electron accelerator or at room temperature.

Ionizing radiation did not cause deterioration of organoleptic properties of mushrooms, on the contrary promoted preservation of aroma and flavour.

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Acta Alimentaria, Vol. 3. (1), pp. 19—25 (1974)

EFFECT OF IONIZING RADIATION ON POST-HARVEST RIPENING PROCESSES OF CULTURED MUSHROOMS *(Agaricus bisporus)*, WITH SPECIAL REFERENCE TO THE RATES OF RESPIRATION AND OF ETHYLENE PRODUCTION

E. Kovács and K. Vas

(Received May 8, 1972)

Recent findings have shown that ionizing radiations considerably prolong the shelf life of edible mushrooms.

Physiological effects of irradiation were studied on respiration and ethylene production of mushrooms.

It was found that

— respiration intensity of radiation treated mushrooms stored in the cold (5—8°C, 70—90% RH) decreased with increasing radiation doses,

— the normally low ethylene production of the mushrooms was increased by irradiation,

— on the basis of respiration studies as well as technological examinations, the keeping time of mushrooms irradiated with 300 krad can be given as 4—5 days, when stored at room temperature.

In Hungary, *Agaricus bisporus* is the most important edible mushroom, now grown on a total area of 250 000 m².

Cultivation and marketing of mushrooms are, however, hampered by their low keeping quality. After harvest, the quality of fresh mushrooms deteriorates rapidly because of post-harvest ripening processes; the cap (pileus) opens, its surface undergoes discoloration and its texture becomes tough (Kovács *et al.*, 1968a).

Studies on prolongation of the shelf life of mushrooms have been conducted in various parts of the world; the methods applied included cooling (Woodmansee, 1964), treatment with chemicals (Halevy & Wittwer, 1965) storage in controlled atmosphere (Sveine *et al.*, 1965), etc.

Treatment by ionizing radiation proved to be a promising new approach (Staden, 1965; MacQueen *et al.*, 1966; Stoller, 1968; Kovács *et al.*, 1968a, b).

Most technological experiments showed good results. The physiological and biochemical effects of irradiation should, nevertheless, be studied in greater detail.

The present studies were carried out to obtain more information on the modification of post-harvest processes as caused by irradiation, on the basis of respiration and ethylene production intensities.

1. Materials and methods

1.1. Raw material

Raw material for measuring oxygen consumption was procured from the Mushroom Production Unit of the "*Duna*" Agricultural Cooperative, Budapest.

CO_2 and ethylene production were determined on commercial mushrooms imported by firms in the German Federal Republic from Holland.

1.2. Radiation treatment

In the oxygen consumption experiments, irradiation was carried out with the panoramic ^{60}Co gamma source (total activity about 80 kCi) of the Institute for Isotopes of the Hungarian Academy of Sciences. The dose rate was varied between 25 and 300 krad $\cdot$ h^{-1}.

The mushrooms used for CO_2 and ethylene determination were irradiated in the Institute for Radiation Technology, Nuclear Research Centre, Leopoldshafen, FRG. The dose rate of the source was 1 Mrad $\cdot$ h^{-1}.

1.3. Determination of oxygen

This was performed according to a method described in an earlier paper (KOVÁCS & VAS, 1974).

1.4. Determination of carbon dioxide and ethylene

2.5 kg mushrooms with closed caps were placed in a 20-litre desiccator in which air was circulated by means of a peristaltic pump. Air flow rate was maintained at 30 ml $\cdot$ min^{-1} to provide a possibly homogeneous air space in the desiccator. Incubation time was 20—24 hours. The gas samples used for analysis were taken directly from the desiccator, on three occasions at 30-minute intervals. The mushrooms were stored at room temperature (21—23°C, 90—95% RH).

Carbon dioxide and ethylene were determined by the quantitative, simultaneous gas chromatographic method of KOVÁCS and VAS (1974).

The analyses were performed in a Hewlett-Packard No. 5750 two-column gas chromatograph.

Detectors: flame ionization detector (for ethylene determination) and heat conductivity detector (for CO_2 determination).

Column: 152.4 cm long stainless steel tube of 0.5 cm inner diameter.

Column packing: *Porapak R* (80—100 mesh).

Carrier gas: helium.

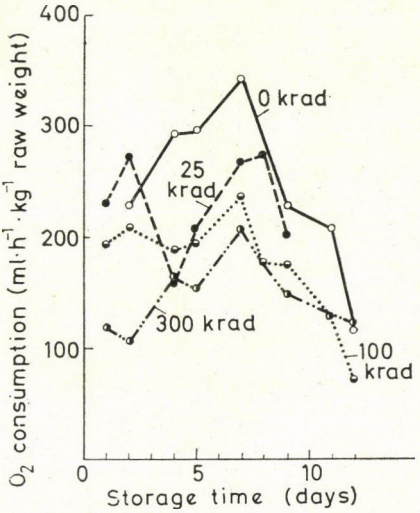

Fig. 1. Respiration intensity of cold-stored mushrooms as a function of irradiation dose and storage time (5—8°C, 70—90% RH, N = 5)

Carrier velocity: 30 ml · min^{-1}: gas pressure at exit was 5 atm gauge pressure.

Column temperature: 50—100°C, with a temperature programme of 10°C · min^{-1}.

Injection port: 75°C.

Flow rate of hydrogen: 25 ml · min^{-1}.

Flow rate of air: 500 ml · min^{-1}.

Temperature of heat conductivity detector: 90°C.

Bridge voltage: 130 mA.

2. Results

The respiration intensity of cold-stored (5—8°C, 70—90% RH) mushrooms was decreased by all applied radiation doses (Fig. 1).

Decrease of respiration intensity with increasing doses can clearly be seen from the figure.

The tendency in the change of respiration intensity of mushrooms stored at room temperature (16—18°C, 65% RH) corresponded to that of cold-stored mushrooms. One day after treatment with 100 krad, the respiration intensity of the sample fell to about half of that measured in the control sample and on treatment with 300 krad it fell still lower (Fig. 2).

Alterations of respiration intensity and ethylene production of mushrooms stored at room temperature (21—22°C, 60—65% RH) are shown in Fig. 3.

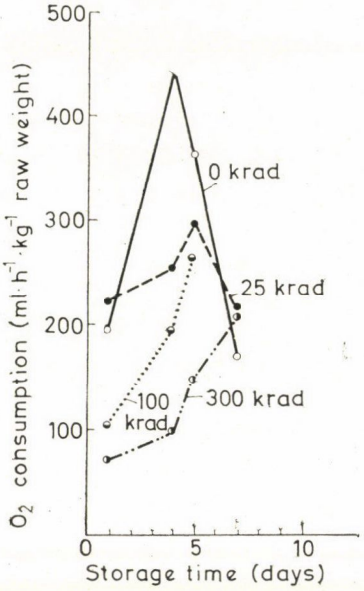

Fig. 2. Respiration intensity of mushrooms stored at room temperature following irradiation with different doses (16—18°C, 65% RH, N = 5)

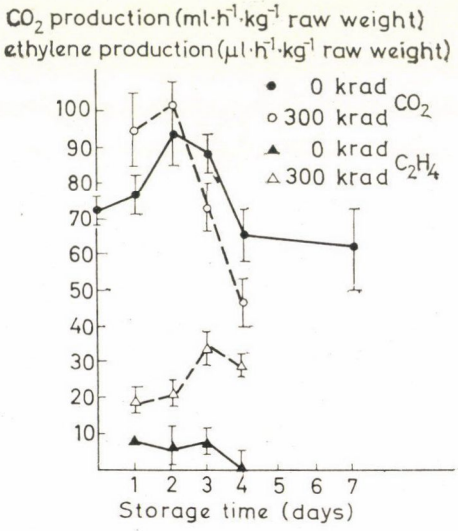

Fig. 3. Respiration intensity and ethylene production of 300 krad-treated and untreated mushrooms as a function of storage time (21—22°C, 60—65% RH, N = 3). The bars indicate standard deviations

3. Conclusions

Ionizing radiation reduced the rate of respiration of mushrooms, as judged on the basis of measurement of oxygen intake. It should be noted that the quality of untreated and radiation-treated mushrooms differed radically after 1 day of storage at room temperature (KOVÁCS *et al.*, 1968a): the caps of the former opened, while those of the latter remained closed. Based on the first results of the respiration studies we inferred that the reduced respiration rate was virtual and might have been due to the greater specific surface of the open caps of the untreated mushrooms, in contrast to the much smaller specific surface of the closed (or scarcely opened) caps of the irradiated specimens.

This interpretation was based on the well known fact that respiration and ethylene production of fruits and vegetables are temporarily, or even lastingly, increased by radiation treatment, which even may elicit an artificial climacterium in certain non-climacteric fruits, *e.g.* citrus species (MAXIE *et al.*, 1966 b).

Respiration studies based on the determination of CO_2 production (Fig. 3) showed that radiation treatment scarcely enhanced the respiration rate of mushrooms and the CO_2 production fell gradually from the 2nd day. It follows from these findings that irradiation retards post-harvest ripening processes in mushrooms and apart from the favourable influence on external and internal quality characteristics, retardation of opening and of discoloration of the caps, preservation of flavour, odour and texture, it also decreases the rate of physiological processes. Evidence that irradiation actually retards ageing is based on the following considerations.

In earlier studies, the maximum keeping time of mushrooms irradiated with 300 krad was assessed as 4—5 days at room temperature (KOVÁCS & VAS, 1974). Maximum shelf life under the above conditions was again found to be 4—5 days on the basis of respiration measurements (Fig. 2). It can be seen from Fig. 2 that the respiration intensity of the 300-krad sample remained at the same level for the first 4 days of storage and tended to increase from the 5th day. Increase of respiration intensity was accompanied by the appearance of the characteristics of ageing (brown discoloration, wilting, change of flavour, odour and texture) except for opening of the cap, since the cap of mushrooms treated with 300 krad did not open at all. It follows that the open or closed state of the cap has no decisive influence on the intensity of respiration.

The follow-up of respiration by CO_2 determination supports the above conclusions, although differences were lower in this case than with oxygen determination. Mention should, however, be made of the fact that other authors (MAXIE *et al.*, 1966a; LEE *et al.*, 1968; ROMANI, 1964, 1966) observed a greater

increase in CO_2-production under the influence of radiation treatment of fruits and vegetables (data on mushrooms are not available).

A qualitative analysis of ethylene production by mushrooms was described by LOCKARD and KNEEBONE (1962), who demonstrated that fungal mycelia produce, apart from carbon dioxide, five other substances: ethylene, acetaldehyde, acetone, ethanol and ethyl acetate. ILAG and CURTIS (1968), who studied the ethylene production of lower fungi, described the intensity of ethylene production by mushrooms as very low. Irradiation enhanced the ethylene production intensity of mushrooms and this tendency corresponded with similar observations on radiation-treated fruits and vegetables.

*

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INVESTIGATIONS INTO THE OSMOTIC RESISTANCE OF PLANT CELL WALLS AND SKINS BY MEANS OF EQUILIBRIUM RELATIVE HUMIDITY DETERMINATIONS

(Preliminary report)

F. HIRSCHBERG

(Received May 15, 1972)

A new approach is suggested for the determination, in physical units, of the resistance (*i.e.* of the water permeability) of the passive anatomic structures (cell wall, skin, etc.) of plants. The method is based on the measurement of the equilibrium relative humidity (water activity) of the part of the plant under investigation and of its cell homogenate. The obtained values are converted into osmotic pressure expressed in atmospheric pressure units and finally the osmotic pressure of the intact part is subtracted from the osmotic pressure value of the homogenate. These conditions were investigated on irradiated and control specimens of the potato varieties: "Gülbaba" and "Rózsa". A significant difference ($P < 0.05$) was found between the water activities of the irradiated potatoes and their tissue homogenates, while there was a non-significant difference in the values of the control (germinated) potatoes.

The method is quick, suitable for individual and serial tests and may offer considerable help in physiological and plant breeding studies. Further work is needed to establish the applicability of the method to animal tissues and micro-organisms.

Biological materials which can be considered aqueous solutions or hydrated aqueous suspensions of complex structure, possess, like other aqueous solutions, a reduced vapour pressure. This means that in a closed space the relative humidity over the biological specimen will be lower than the vapour pressure (humidity) in the atmosphere over pure water of the same temperature. While this reduced vapour pressure can be calculated by means of Rault's law for solutions of several simple substances of known molecular weight and readily soluble in water (*e.g.* common salt, sugar, low carbon number alcohols, oxo-compounds, organic acids, etc.), provided their concentration is known, in case of complex biological substances this has to be determined experimentally.

Reduced vapour pressure is expressed either as equilibrium relative humidity (*ERH*):

$$ERH = \frac{P_{soln.}}{P_{H_2O}} 100\,(\%) \tag{1}$$

where

$P_{soln.}$ = vapour pressure of the solution of the substance at a given temperature;

P_{H_2O} = saturation vapour pressure of water at the same temperature; or as water activity (a_w)

$$a_w = \frac{P_{soln.}}{P_{H_2O}} . \tag{2}$$

It can be seen that, numerically, water activity is one hundredth of the equilibrium relative humidity

$$a_w = ERH \cdot 10^{-2}. \tag{3}$$

JOHN and SEKHON (1968) published a detailed survey of the instruments with which *ERH*, that is a_w, can be determined. The following correlation is valid between water activity and the equally concentration dependent osmotic pressure:

$$\pi = \frac{-RT \ln a_w}{\overline{W}}$$

where

π = osmotic pressure (atm),
R = universal gas constant (atm/°K · mole),
T = temperature (°K),
$\overline{W}$ = water content in mole fraction.

The water activity of biological substances is highly important from the physiological point of view. ACKER (1969) proved enzymic reactions to have water activity optimum or limits (*e.g.* lipase, etc.). The anabiotic state of reproductive bodies (*e.g.* seeds, spores) is the result of reduced water activity due to low moisture content which brings about reduced enzyme activity.

The problem is further complicated by the morphological properties of biological substances, in particular by their cellular structure and the additional formations of cells. In the case of plants the cell membrane and the laminar cell wall already constitute a considerable resistance to which thickening of the cell wall, the cuticles, incrustations contribute as further sources of "resistance". All these have to be taken into consideration when measuring the *ERH* of some part of the plant.

The osmotic pressure, *i.e.* the water activity of cells, and the gradient of these properties play a decisive role in the absorption of water from the soil and in the extravascular stages of water and assimilate transport. This explains the great importance of water activity in physiology.

The role of *ERH* is of great importance in storage practice, too. If we wish to preserve the texture, moisture content, anabiotic state, etc. of the stored vegetable part, then, in addition to optimum temperature, an appropriate equilibrium relative humidity must be maintained. Several authors have investigated the relationship between storage and a_w (HEISS & EICHNER, 1971a, b).

CAURIE (1970) suggested a mathematical formula for the safe moisture content of stored products:

$$\frac{1}{M} = K \ln C - a_w \ln r, \tag{5}$$

where

M = safe moisture content of the product (water/solids),
K = slope of the sorption curve in the coordinate system $ln\ C$ vs. a_w,
C = concentration of the substance (solids/water),
a_w = water activity,
r = exponent.

Storability may be related to many factors of which the water activity of the product plays a decisive role, while this latter depends partly on physiological characteristics (age, place and year of growing, etc.) and on anatomic conditions (cell wall, hairiness, cuticle, etc.) these last being properties indicative of the species or of even lower taxonomic categories.

In plant breeding these factors often appear in a complex manner. For instance, the good or poor storability of some potato varieties might be of physiological origin (due *e.g.* to a more intensive metabolism of the variety or clon), but might also be the result of some factors of morphological nature (closed epidermis, quality of the cell cuticle, etc.). If these are known, it will be possible to select the parent or determine the direction of clon selection.

It is considered that the *ERH* test might be suitable for the study of problems of this type. In this paper the results of preliminary investigations are reported on.

1. Materials and methods

1.1. Potatoes

1.1.1. Potato variety "Gülbaba".

1.1.1.1. Irradiated potatoes. — Samples were obtained from the Pilot Food Irradiator of the Central Food Research Institute. Dose applied: 8 krad, date of irradiation: first half of January, 1972. Date of tests: February 15 to March 10, 1972. Generally, at the time of testing no sprouting of the samples was observed, however, a few tubers showed small sprouts (of a few mm length).

1.1.1.2. Control potatoes. — Samples of the same origin. At the time of the test sprouts of about 1 cm length were on the tubers.

1.1.2. Potato variety "Rózsa".

1.1.2.1. Irradiated potatoes. — Samples were obtained from the Radiology Department, Pilot Food Irradiator of the Central Research Institute. Dose: 8 krad, date of irradiation: first half of January, 1972. The samples were tested

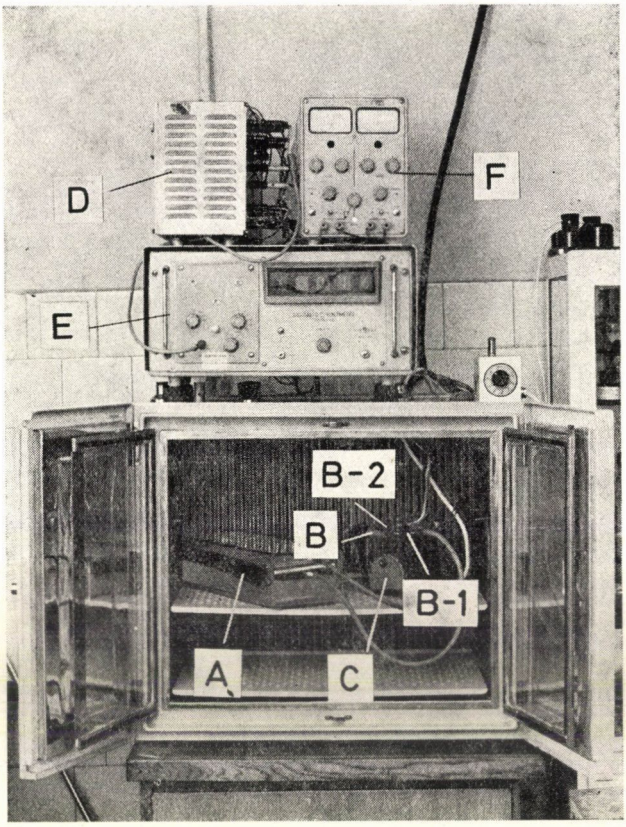

Fig. 1. *ERH* apparatus. A — measuring chamber; B_1 — resistance thermometer; B_2 — lithium chloride probe; C — membrane pump; D — measuring bridge; E — digital tube voltmeter; F — stabilized supply unit

between February 15 and March 10 of the same year. Generally at the time of the tests some of the tubers had small sprouts (of a few mm length).

1.1.2.2. Control potatoes. — The samples were of the same origin. At the time of the test there were sprouts of about 1 cm length on the tubers.

1.2. Instruments

1.2.1. Apparatus for the determination of ERH. Instrument for the determination of dew point, provided with a LiCl probe (for details see HIRSCHBERG & SZABÓ, 1972). A photograph of the apparatus is presented in Fig. 1, its principle of operation is shown in Fig. 2.

The apparatus is calibrated with saturated salt solutions, using YOUNG's (1967) table. The vapour pressure values pertaining to different temperatures were read off the graphs published by HENGSTENBERG and co-workers (1957).

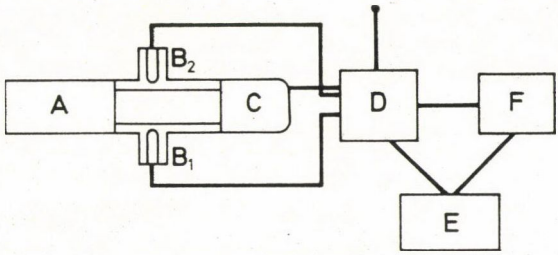

Fig. 2. Schematic diagram of the *ERH* apparatus. A — measuring chamber; B_1 — resistance thermometer; B_2 — lithium chloride probe; C — membrane pump; D — measuring bridge; E — digital tube voltmeter; F — stabilized supply unit

1.2.2. "Komet" domestic kitchen machine. (Manufactured in the GDR.) The turmix blendor belonging to the apparatus was used to homogenize the potato tubers.

1.3. Methods

1.3.1. ERH test of whole potatoes. 200- to 250-g samples of whole potatoes were placed into the measuring chamber and the circulation of air continued until equilibrium was reached, that is the value shown by the millivoltmeter remained constant for at least 10 minutes.

1.3.2. ERH test of potato tissue homogenates. Disruption of the potato cells appeared to be a considerable problem. Mechanical methods, such as homogenizing with the "Turmix" blendor were accompanied by a significant rise in temperature which interfered with the measurements. Finally a method was adopted which consisted in placing the whole potatoes into the deep-freezer of a Saratow type refrigerator. The frozen potatoes were film-wrapped and allowed to thaw. (Wrapping was necessary to avoid the precipitation of water from the atmosphere on the surface of cold potatoes.) After thawing, the potatoes were considerably softened and dripped, since the ice crystals had destroyed a considerable part of the cells (similar histological tests have been described by MOHR, 1971). The potatoes were put into the turmix blendor while still cold and were homogenized. Homogenization was not accompanied by a substantial increase of the temperature. The microscopic picture of the homogenate in Fig. 3 shows the presence of free starch particles, many cell fragments and only relatively few intact cells. 200 to 250 g of the homogenate were placed into the measuring chamber and the *ERH* value determined.

The water activity of both the whole tubers and of the homogenates was measured at 25 $\pm$ 2°C, the volume of the measuring chamber was about 900 ml, the rate of air circulation was about 2 l/min.

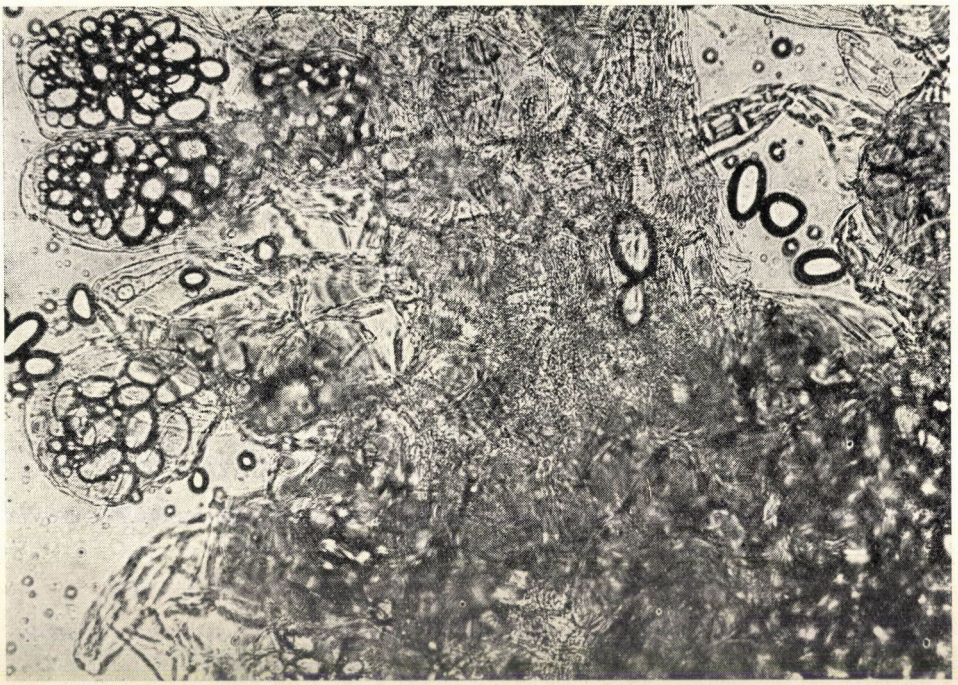

Fig. 3. Microscopic picture of the tissue homogenate of potato tubers

2. Results

The equilibrium relative humidity of irradiated and control samples of the potato varieties "Gülbaba" and "Rózsa" was determined in six replicates. After the determination of *ERH* the tubers were frozen and thawed and used for the preparation of tissue homogenates. Finally, the *ERH* of the homogenates was also determined.

From the *ERH* values, the true osmotic pressure of the homogenates and the "calculated" osmotic pressure of the whole potato tubers were calculated with the help of Eq. 4. The results are summarized in Table 1.

It appears from the table that tissue homogenates possess the highest water activity, while the water activity of germinated controls is lower and that of the irradiated tubers the lowest. There is a certain difference in the values obtained for identically treated Gülbaba and Rózsa varieties, but these differences are not significant. The fact that some of the treated (irradiated) potato tubers showed signs of sprouting interfered with the evaluation of the results, mainly in the case of the Gülbaba variety.

3. Conclusions

From the results the following conclusions were drawn:

- There are significant differences (at the 95% probability level) between the water activities of irradiated whole tubers and tissue homogenates.
- The difference between the water activities of control (germinated) tubers and tissue homogenates is not significant.
- There is no significant difference with respect to water activity between potato varieties identically treated (untreated or irradiated), though the water activity and osmotic pressure values of the Gülbaba variety are higher.
- By converting water activity data to osmotic pressure values it seemed possible to determine the resistance of the passive anatomical protective structures (skin and cell walls) in physical units with the help of the equation:

$$P_r = \pi_h - \pi_i \text{ (atm)}, \tag{6}$$

where

P_r = resistance of the passive anatomical protective structures (atm),
π_h = osmotic pressure of the homogenate (atm),
π_i = calculated osmotic pressure of intact tubers (atm).

- The different P_r values of potato tubers might be attributed, in first approximation, to the higher resistance of the suberized skin of the irradiated tubers compared to the resistance of the young thinish epidermal cells or cell walls of the sprouts of the controls. Of course, other factors, such as respiration, metabolism and other effects of irradiation may contribute to the development of this phenomenon.

The method enables the easy and rapid determination of the resistance, that is of the permeability of the skin and cell wall and permits of gaining important information in the fields of plant physiology, plant breeding, etc.

The method is presumably not limited to the investigation of plant organisms, but may be extended to the cells of animal tissues and of microorganisms, but further work is required before this can be confirmed.

When applying this method, great care has to be taken to avoid water losses during the digestion of the cells and any other damage to cell texture (*e.g.* thermal coagulation, enzymic degradation, etc.).

It is also obvious that the method furnishes average values, hence the method allows no differentiation between the various kinds of tissues (parenchimal, transport, etc.) of which the organ (in our case the potato tuber) consists. Nevertheless, the experimental results obtained in this manner may provide valuable information on the resistance of cell walls, cell membranes, skin tissues, information which hitherto could only be gained by highly intricate procedures, or not at all.

Table 1

ERH tests

	Irradiated								
	Whole tuber				Cell homogenate				$\pi_1-\pi_2$ atm
	V_{H_2O}	V_{LiCl}	a_w	π_1 atm	V_{H_2O}	V_{LiCl}	a_w	π_2 atm	
Potato variety		0.0519	0.725	4.05		0.0552	0.860	1.92	
Rózsa		0.0519	0.725	4.05		0.0558	0.880	1.63	
		0.0518	0.720	4.26		0.0559	0.885	1.56	
		0.0517	0.715	4.23		0.0551	0.855	2.00	
	0.0179	0.0519	0.725	4.05	0.0179	0.0552	0.860	1.92	
	a	0.0542	0.815	2.73		0.0549	0.845	2.28	
mean	—	—	0.737	3.89	—	—	0.864	1.88	2.01
standard deviation			±0.04	±0.60			±0.02	±0.23	
Potato variety	a	0.0544	0.825	2.44		0.0562	0.900	1.34	
Gülbaba	a	0.0544	0.825	2.44		0.0562	0.900	1.34	
		0.0519	0.725	4.05		0.0559	0.855	1.55	
		0.0519	0.725	4.05		0.0555	0.870	1.64	
		0.0519	0.725	4.05		0.0559	0.885	1.55	
	0.0179	0.0519	0.725	4.05	0.0179	0.0559	0.885	1.55	
mean	—	—	0.758	3.51	—	—	0.882	1.49	2.02
standard deviation			±0.05	0.83			±0.02	±0.12	

a: slightly sprouting
b: strongly sprouting

V_{H_2O} = voltage of the resistance thermometer
V_{LiCl} = voltage of the lithium chloride probe
π = osmotic pressure
a_w = water activity

*

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on potatoes

Control								
Whole tuber				Cell homogenate				$\pi_1 - \pi_2$ atm
V_{H_2O}	V_{LiCl}	a_w	π_1 atm	V_{H_2O}	V_{LiCl}	a_w	π_2 atm	
	b 0.0548	0.840	2.23		0.0551	0.855	1.99	
	b 0.0549	0.845	2.16		0.0552	0.860	1.92	
0.0179	b 0.0548	0.840	2.23	0.0179	0.0553	0.865	1.84	
	b 0.0548	0.840	2.23		0.0559	0.885	1.55	
	b 0.0548	0.840	2.23		0.0559	0.885	1.55	
	b 0.0548	0.890	2.23		0.0559	0.885	1.55	
—	—	0.84)	2.22	—	—	0.872	1.73	0.49
		0.0)	±0.03			0.01	0.22	
	b 0.0553	0.865	1.84		0.0557	0.880	1.64	
	b 0.0555	0.870	1.64		0.0555	0.870	1.64	
0.0179	b 0.0559	0.885	1.55	0.0179	0.0556	0.875	1.70	
	b 0.0559	0.885	1.55		0.0559	0.885	1.55	
	b 0.0542	0.815	2.60		0.0571	0.935	0.87	
	b 0.0554	0.865	1.86		0.0571	0.935	0.87	
—	—	0.864	1.84	—	—	0.896	1.38	0.46
		0.02	0.3)			0.03	0.39	

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REDUCTION OF THE PROTEOLYTIC ACTIVITY OF A MILK CLOTTING ENZYME PREPARATION FROM A *MUCOR* STRAIN

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The aim of this study was to purify the crude enzyme preparation produced by surface fermentation of *Mucor pusillus* Lindt.

20—40% of the crude enzyme preparation is a water insoluble sediment, its milk clotting enzyme content is 20 000—40 000 U/g and its proteolytic activity, related to clotting activity, is 5 to 6 times that of calf rennet. Fractionated precipitation of the enzyme solution was carried out with ethanol, acetone and ammonium sulphate at various saturation levels. Fractionated precipitation with ammonium sulphate proved to be most suitable for the purpose because it enriched the enzyme content and at the same time separated, to a certain degree, milk clotting and proteolytic activities. The enzyme preparation thus obtained is completely water soluble and has a milk clotting activity of 104 000 U/g and its proteolytic activity related to the milk clotting activity is about 3.5 times higher than that of calf rennet. The quality of the preparation equals that of Meito-rennet, made in Japan.

The specific activity of the enzyme was further increased and the relative proteolytic activity was reduced by applying the fractionated concentrate to an Amberlite CG—50 cation exchange column. The milk clotting activity of the preparation thus obtained is about 7fold as compared to the enzyme fractionated with ammonium sulphate and about 35fold as compared to calf rennet. The proteolytic activity related to the milk clotting activity is still about twice as high as that of calf rennet. However, according to the observations of Japanese researchers, no further reduction of proteolytic activity is possible.

Cheese manufacturing experiments were carried out with enzyme preparations of various purity grades and it was concluded that the preparation obtained by fractionation with ammonium sulphate is satisfactory for the purpose.

Experiments have been conducted in this Institute on the preparation of microbial milk-clotting enzymes of rennin-like activity, in order to substitute calf rennet, at least partly, in cheese production, because rennet is in short supply all over the world.

Two firms, Meito Sangyo Ltd., Japan, and the Pfizer Co., USA, have each marketed a milk clotting enzyme preparation in 1967. Meito-rennet is prepared by surface fermentation of *Mucor pusillus* Lindt, Sure Curd by submerged fermentation of the mould strain *Endothia parasitica*. Favourable results obtained on testing these preparations in cheese manufacture initiated investigations on such enzymes in Hungary.

Curdling enzyme preparations from moulds differ from calf rennet in several aspects (VÁMOS *et al.*, 1969a), *e.g.* in their Ca^{++}-sensitivity, higher proteolytic activity and production of softer curd. Occasionally cheeses produced

with microbial enzymes have a slightly bitter after-taste. Since these characteristics seemed to result mainly from the relatively higher proteolytic activity as compared to clotting power in the microbial enzyme preparations, an assay to improve their quality through reduction of proteolytic activity was indicated. This paper deals with the purification of a crude enzyme preparation obtained by surface fermentation of *Mucor pusillus* Lindt (VÁMOS *et al.*, 1969b).

1. Materials and methods

1.1. Enzyme preparations

E3 is a crude curdling powder prepared in this Institute by surface fermentation of the mould strain *Mucor pusillus* Lindt, cultivated on wheat bran. 20—40% of the enzyme powder is a water insoluble sediment, its milk clotting enzyme content is 20 000—30 000 U/g and its proteolytic activity, related to clotting activity, is 5—6 times higher than that of calf rennet. The more complete decomposition of proteins brought about by the higher proteolytic activity is probably responsible for the bitterish aftertaste occurring in cheeses prepared with Mucor-rennet (TOKITA, 1969).

Meito-rennet is a microbial milk clotting powder prepared from surface cultures of the mould *Mucor pusillus* Lindt (Meito Sangyo Corporation, Japan). It is completely soluble in water and its milk clotting enzyme content is 115 000 U/g. Its proteolytic activity related to clotting activity is twice as high as that of calf rennet.

Powdered calf rennet hereafter referred to as rennet, was produced from partly imported calf stomach (Húsipari Készletező és Göngyölegellátó Vállalat, Budapest).

1.2. Activity measurements

1.2.1. Measurement of milk clotting activity. Milk clotting activity was determined in 10% rehydrated milk powder and expressed in Soxhlet units (SOXHLET, 1877). Rehydration was carried out in tap water instead of $CaCl_2$ solution, because Ca^{++} had previously been found to differently activate the clotting power of milk clotting preparations of various origin and purity (POZSÁR *et al.*, 1970). The number of units of activity corresponds to the volume of milk (ml) coagulated by 1 g enzyme in 40 minutes at 35°C.

1.2.2. Measurement of proteolytic activity. Proteolytic activity was determined on the basis of casein decomposition. The number of activity units corresponds to the amount of casein (mg) decomposed by 1 g enzyme in 1 hour at pH 6 and 35°C (VÁMOS *et al.*, 1969c).

1.3. Characterization of the preparation

To characterize enzyme preparations of different degrees of purity, in addition to their milk clotting and proteolytic activities, the quotient of the latter two values ("H") and their specific activity were also calculated.

1.3.1. Determination of specific activity. Specific activity is understood to be the quotient of milk clotting activity and optical density (extinction) at 280 nm of the enzyme solution. Increase of the specific activity indicates progress in the purification of the enzyme protein.

1.3.2. Determination of the quotient "H". The value "H" is the quotient of the clotting and proteolytic activities of the enzyme, viz. the number of units of clotting activity per unit proteolytic activity. The increase of the "H" value indicates decrease in the relative proteolytic activity of the preparation.

According to previous paper electrophoretic studies the reduction of proteolytic activity of the crude enzyme preparation seemed to be possible, because two zones of proteolytic activity were obtained only one of which coincided with the zone of clotting activity (VÁMOS & MORVAI, 1970). Reduction of the proteolytic activity was attempted by

a) fractionated precipitation,
b) change of pH,
c) separation by ion exchange.

1.4. Fractionated precipitation of the crude enzyme solution

Fractionated precipitation was carried out with ethanol, acetone and ammonium sulphate, using a 15% solution of crude enzyme. The applied respective saturation degrees were 45, 50, 55, 60, 70 and 75% with acetone, 40, 50, 60, 70 and 80% with ethanol and 40, 50, 60 and 70% with ammonium sulphate. The precipitates obtained in the saturation ranges were collected by centrifugation and dissolved in a small amount of distilled water. Each enzyme solution thus obtained was tested for milk clotting and proteolytic activities and its optical density was measured at 280 nm. The activity and extinction values thus obtained were expressed as % of total recovered activity and extinction and the total recovered clotting activity was related to the activity of the original solution.

1.5. Separation of milk clotting and proteolytic activities by ion exchange

Since the ratio of proteolytic to clotting activity could not be reduced beyond a given limit by fractionated precipitation purification by ion exchange was attempted on the basis of relevant literature (IWASAKI *et. al.*, 1967; ARIMA

et al., 1968). Columns were packed with Amberlite CG—50 cation exchanger, regenerated and equilibrated with the buffer solution before use.

The enzyme solution was applied to the column thus prepared and eluted by a stepwise procedure. Elution was commenced with 0.05 *M*, pH 3.5 Na-acetate buffer and was continued with 0.2 *M* pH 5.0 Na-acetate buffer. The purified enzyme was eluted with the latter from the column. Since the enzyme solution was considerably diluted by the ion exchange purification procedure, the enzyme proteins were collected from the fractions by precipitation with ammonium sulphate (at 60% saturation) and the centrifuged precipitate was dissolved in a minimum amount of distilled water.

The purified, concentrated enzyme solution was passed through a Sephadex G—25 column to remove sulphates and then freeze-dried, or precipitated with a threefold volume of acetone and dried with absolute alcohol. In the latter case, precipitation of the purified enzyme required the addition of 1 or 2 drops of a $CaCl_2$ solution. Dialysis was not suitable for the removal of sulphates, because the cellulase impurity of the enzyme damaged the dialyzing membrane.

2. Results

2.1. Fractionated precipitation of the crude enzyme solution

Separation of the milk clotting and proteolytic activities of the crude enzyme solution was attempted by precipitation with acetone, ethanol and ammonium sulphate solutions of different concentrations. The total milk clotting activities recovered after precipitation expressed as per cent of the initial activity, were as follows:

50% with ethanol,
80% with acetone,
90—95% with ammonium sulphate.

Only analytical grade acetone was suitable for fractionation, since technical grade acetone inactivated the enzyme.

Distribution of the activities recovered and extinction values measured in the ranges between saturation steps of fractional precipitation can be seen from Table 1. All values are expressed as per cent of total recovered activity.

As seen from the Table this method is not suitable to separate the two enzymes, no substantial change was caused in their proportion either, however, the amount of non-enzymic impurities (colouring matter, mycelia and bran residues) was considerably reduced.

Fractionated precipitation with ammonium sulphate proved to be most suitable for the purpose, not only because technical grade ammonium sulphate

Table 1

Purification of dissolved crude enzyme powder E3 by fractionated precipitation
(E3 was prepared from surface cultures of *Mucor pusillus* Lindt)

a) Fractionation with ethanol

Saturation	Distribution of activity in precipitate expressed as per cent of total recovered activity		H
	Milk clotting activity	Proteolytic activity	
50%	6%	11%	9.3
50–60%	8%	12%	11.3
60–70%	85%	75%	19.3
70% (no precipitate)	1%	2%	6.7

b) Fractionation with acetone

Saturation	Distribution of activity and extinction in fractions expressed as per cent of total recovered activity and extinction			H
	Milk clotting activity	Proteolytic activity	Extinction at 280 nm	
45%	2%	6%	25%	8.2
45–60%	80%	70%	70%	21.6
60–75%	15%	19%		15.0
75% (no precipitate)	3%	5%	5%	7.5

c) Fractionation with ammonium sulphate

Saturation	Distribution of activity and extinction in fractions expressed as per cent of total recovered activity and extinction			H	Specific activity
	Milk clotting activity	Proteolytic activity	Extinction at 280 nm		
40%	15%	29%	42%	8.5	19
40–50%	54%	41%	18%	24.7	174
50–60%	28%	24%	12%	20.0	140
60–70%	3%	6%	5%	8.9	46
70% (no precipitate)	—	—	23%	—	—

H: quotient of values for milk clotting and proteolytic activities.
Specific activity: quotient of milk clotting activity and extinction at 280 nm

could be utilized, but also because the precipitate formed in the range between 40 and 60% saturation contained the bulk of the milk clotting activity of the original crude enzyme. Crude enzyme preparations of different quality were treated with ammonium sulphate and the precipitate formed in the 40 to 60% saturation range was dissolved and passed through a Sephadex G—25 column to remove salts. The enzyme solution thus purified was either freeze-dried or treated with a threefold volume of acetone to precipitate the enzyme.

Independently of the original enzyme preparation the quality of the end-products was the same: they were completely water soluble and had a milk clotting enzyme content of 104 000 U/g, which was 2.5—5 times higher than that of the original crude enzyme. Their proteolytic activity related to milk clotting activity was about 3.5 times higher than that of calf rennet.

Milk clotting and proteolytic enzyme activities as well as total extinction at 280 nm for end products of three crude enzyme preparations of different quality are compared in Table 2.

Table 2

Activity and extinction values of preparations obtained by purification of different crude enzymes with ammonium sulphate, expressed as per cent of the initial values

Activities recovered in end product	Basic enzyme preparations		
	Twice precipitated with isopropyl alcohol	Crude I	Crude II
Milk clotting activity	66.5%	67.4%	65.4%
Proteolytic activity	61.4%	44.4%	32.1%
Extinction at 280 nm	27.6%	18.0%	12.4%
Specific activity	115	133	127
H	23.0	24.3	24.7

H: quotient of values for milk clotting and proteolytic activities.
Specific activity: quotient of milk clotting activity and extinction at 280 nm

As can be seen, the end products of all three crude preparations contained two thirds of the original milk clotting activity. The recovered proteolytic activity and the extinction value at 280 nm were the lower the more impurities had been present in the original crude preparation.

Fractionation with ammonium sulphate of the crude preparation of poorer quality not only enriched the enzyme content, but also separated milk clotting and proteolytic activities to a certain degree.

Data of crude enzyme preparations of different quality and of the end products obtained from them by fractionation with ammonium sulphate are shown in Table 3.

Table 3

Data of crude enzyme preparations and their respective end products obtained by fractionation with ammonium sulphate

Enzyme preparation	Milk clotting activity U/g	Proteolytic activity U/g	H	Specific activity
Twice precipitated with isopropanol	43 000	2 450	17.6	67
Crude I	29 300	1 800	16.4	59
Crude II	19 000	1 350	14.0	22
Ammonium sulphate fractionation product	104 000	4 200	24.7	130

H: quotient of values for milk clotting and proteolytic activities.
Specific activity: quotient of milk clotting activity and extinction at 280 nm.

Direct fractionation of the crude fermentation liquor with ammonium sulphate was also attempted; a highly active and entirely water soluble substance was thus obtained without methanol precipitation.

Experiments were also performed to clarify whether repeated precipitation with acetone or ammonium sulphate can further increase the proportion of milk clotting activity related to proteolytic activity. Repeated precipitation did not, however, alter the ratio of the two kinds of activity.

Attempts to influence, by changing the pH, the ratio of milk clotting to proteolytic activity in enzyme solutions purified by fractionation with ammonium sulphate also failed.

Acidification of the enzyme solution to pH 3 and neutralization after periods of 2, 15, 30 and 60 minutes did not affect its enzyme activity either. Acidification to pH 2.5 and pH 2 reduced both activities by 13 and 40%, respectively.

2.2. Experiments into separation of proteolytic and milk-clotting activities by ion exchange

As fractionated precipitation failed to separate milk clotting and proteolytic enzyme activities beyond a certain degree, ion exchange was employed for further purification.

The enzyme previously purified by fractionation with ammonium sulphate was passed through an Amberlite CG—50 cation exchange column and collected from it by stepwise elution. This procedure removed a part of the colouring substances and the specific activity was considerably increased;

although the clotting and proteolytic activities could not be separated, the ratio of proteolytic to milk-clotting activity could be further reduced, to almost half of that measured in the end product of fractionation with ammonium sulphate. The main data of purification by ion exchange are summarized in Table 4 and illustrated in Fig. 1.

Table 4

Purification of the enzyme preparation E3 from surface fermentation by ion exchange on Amberlite CG—50 column

Elution	Distribution of		
	extinction at 280 nm	milk clotting activity	proteolytic activity
	expressed as % of initial values		
in 0.05 *M* pH 3.5 Na-acetate buffer	45% $s = \pm 3.9$	10% s = ±5.9	28% s = ±2.0
in 0.2 *M* pH 5.0 Na-acetate buffer	27% s = ±3.5	85% s = ±8.5	45% s = ±3.2
Bound by column	28% s = ±4.2	5%	27%

n = number of measurements (5—8)
s = standard deviation

The first buffer (0.05 *M* pH 3.5 Na-acetate) eluted from the column 45% of the colouring matter responsible for the original extinction along with about 10% of the amount of the milk clotting enzyme applied to the column. The second buffer (0.2 *M* pH 5.0 Na-acetate) eluted about 85% of the original clotting activity, and about 45% of the proteolytic activity. About 5% of the original milk-clotting activity, 27 to 28% of the proteolytic activity and of the colouring matter responsible for extinction were bound by the column.

Enzyme proteins were precipitated from the second eluate by 60% saturation with ammonium sulphate, the precipitate was dissolved in a small amount of distilled water, the concentrated enzyme solution thus obtained was passed through a Sephadex G—25 column to remove salts and finally it was either freeze-dried or precipitated with a threefold volume of acetone. In the latter case 1 or 2 drops of $CaCl_2$ had to be added to elicit the reaction. The final purified product contained 700 000 U/g milk clotting enzyme.

Attempt was made at a second purification by ion exchange by passing the product of the first purification through an Amberlite CG—50 column again following precipitation with ammonium sulphate. The active enzyme was eluted again by the second buffer solution.

The specific activity of the twice purified enzyme increased further, but the ratio of milk clotting and proteolytic activities did not change.

Data of the different enzymes prepared in this laboratory are summarized in Table 5, with the corresponding data for calf rennet and Meito-rennet presented for comparison.

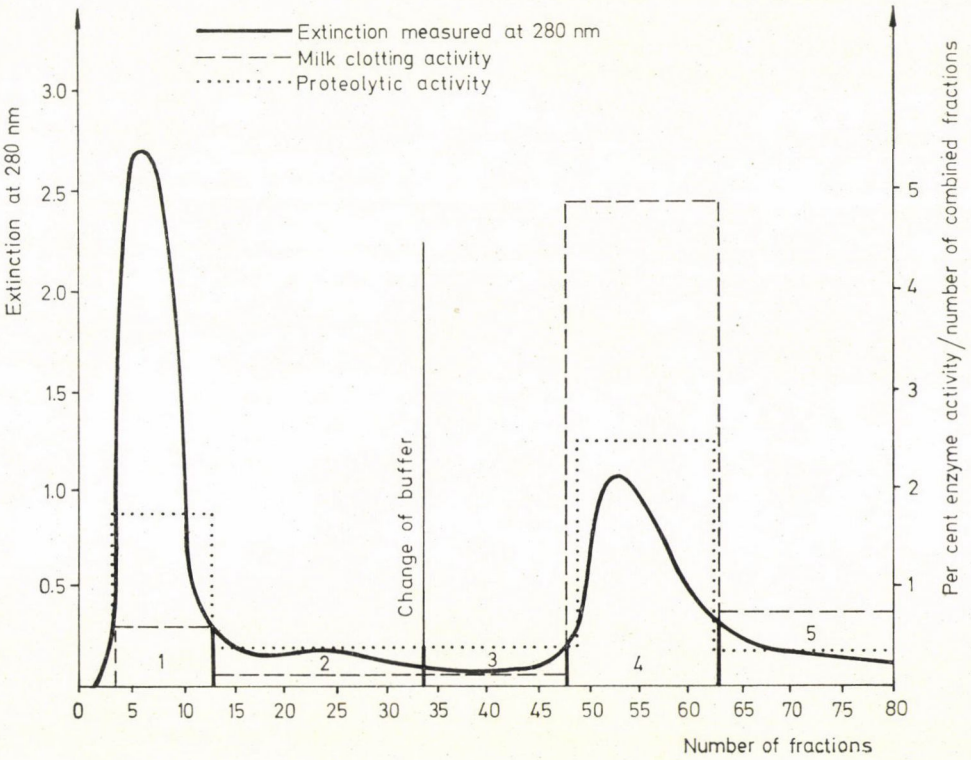

Fig. 1. Purification of the enzyme preparation E3 on a column of Amberlite CG—50. Fractions 1, 2, 3, 4 and 5 combined for activity measurement

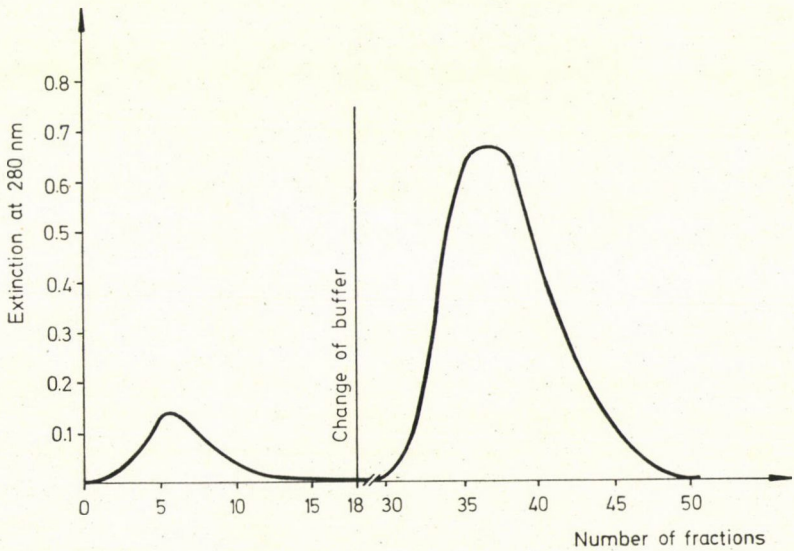

Fig. 2. Repeated purification of the enzyme preparation E3 on a column of Amberlite CG—50

Table 5

Data of enzyme preparations of different purity

Preparation	Milk clotting enzyme content U/g	Proteolytic enzyme content U/g	H	Specific activity
Crude enzyme	24 000	1 600	15.0	41
Ammonium sulphate fractionation	104 000	4 200	24.7	140
Once purified by ion exchange	730 000	17 500	41.7	570
Twice purified by ion exchange	760 000	18 000	42.2	760
Meito rennet	115 000	3 000	38.3	230
Calf rennet	19 000	222	85.5	365

H: quotient of milk clotting and proteolytic activities.
Specific activity: quotient of milk clotting activity and extinction measured at 280 nm.

As can be seen, the ratio of proteolytic and milk clotting activities of the ion exchange purification product was twice as high as that of calf rennet and equal to, or slightly inferior to, that of Meito-rennet. The milk-clotting enzyme concentration of the preparation was about 6, 7 and 35 times higher than that of Meito-rennet, that of the ammonium sulphate purification product and that of calf rennet, respectively.

After fractionation with ammonium sulphate, subsequent purification by ion exchange, removal of salts and precipitation, the end product contained about 30% of the initial milk clotting activity.

3. Conclusions

As already noted, the ratio of proteolytic to milk clotting enzyme activity as measured in our fungal enzyme preparation was twice as high as in calf rennet. According to Japanese authors ((ARIMA *et al.*, 1968) this ratio is, even in crystalline Mucor-rennet produced by these authors, 1.6 fold of that in calf rennet. It follows that no notable reduction of relative proteolytic activity, viz. increase of the "H" value can be reasonably expected from further purification of our preparation. Proteolytic action is an initial step in the mechanism of milk clotting by splitting a peptide molecule from casein, thereby enabling the formation of paracasein. To decide whether the higher proteolytic activity of the microbial enzyme preparation would interfere with its use in cheese production, both ammonium-sulphate-purified and ion-exchange-purified enzymes were prepared in amounts sufficient to test in the production of an experimental batch of portsalut cheese. The experiments were carried out in the Experimental Plant of the Hungarian Dairy Research Institute, Mosonmagyaróvár, and in the Training Workshop of the School of Dairy Technology,

Csermajor, with the usual technology, using Mucor enzyme preparations purified by fractionated precipitation with ammonium sulphate. From a part of the preparation used in the experiments sulphate was removed (KISS & GAJZÁGÓ, 1970).

Although rank sums for the general quality of the control samples manufactured with calf rennet were significantly higher than those of samples prepared with fungal rennet, the difference in taste and texture scores was not significant.

It is, therefore, hoped that the difference can be further decreased by certain modifications of production technology. The experimental observations indicate that purification of the crude enzyme by ion exchange is not necessary for cheese production purposes, because fractionated precipitation with ammonium sulphate can in itself remove enough impurities to eliminate the bitter aftertaste.

Since surface fermentation is less convenient on an industrial scale than is submerged fermentation, the latter possibility has also been studied. But the submerged cultures of the same mould strain synthetized an enzyme of different composition having a 10—20 times higher relative proteolytic activity than the crude enzyme from surface cultures. Experiments to reduce proteolytic activity in the enzyme preparation gained from submerged cultures are in progress.

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STUDIES INTO THE o-DIPHENOL OXIDASE ACTIVITY OF POTATOES

PART II. — SOME CHARACTERISTICS OF THE ENZYME, ITS AMOUNT IN DIFFERENT VARIETIES AND ITS CHANGES ON STORAGE

L. VÁMOS-VIGYÁZÓ and N. KISS-KUTZ

(Received June 26, 1972)

The activity assay method described in a preceding paper (VÁMOS *et al.*, 1973) was applied to determine o-diphenol oxidase activity in the different parts of the potato tuber, its changes with temperature, with pH and on storage of 3 varieties marketed in Hungary, as well as its value in 8 experimental varieties destined for processing.

The main results are as follows:

1. Enzyme activity follows the Arrhenius equation in the temperature range from 25 to 35°C (Figs. 1, 2).
2. In the pH-range from 6.2 to 7.3 activity increases according to an exponential plot (Fig. 3).
3. The tissue around the "eyes" of the tuber contains, in spite of its higher enzyme concentration, only about 4 per cent of the total enzyme content of the peeled potato (Table 1).
4. Differences in enzyme content between lots of the same variety were of the same order as between lots of different varieties (Fig. 6).
5. Initial differences between the enzyme concentrations of three commercial varieties decreased considerably by the 60th or 70th day of winter storage at +5°C. On further storage for a similar period only minor fluctuations in enzyme concentration could be observed, in spite of sprouting occurring during the last month (Fig. 4).
6. The experimental potato varieties tested had significantly lower o-diphenol oxidase enzyme concentrations than the great majority of the lots investigated of commercial varieties (Figs. 5, 6).

The activity assay method described in Part I of this paper (VÁMOS-VIGYÁZÓ *et al.*, 1973) was applied to study the *in situ* characteristics of the enzyme o-diphenol oxidase in some Hungarian potato varieties and experimental varieties, respectively. The investigations include establishing enzyme distribution in the potato tuber, changes in activity with temperature, with pH and on cold storage, as well as determining the variation of enzyme content in different lots of the same variety.

1. Materials and methods

1.1. Potatoes

The varieties Gül baba, Ella and Rózsa were used throughout the experiments as well as 8 experimental varieties destined for manufacturing deep-frozen chips.

1.2. Determination of enzyme activity

Enzyme activity was measured according to the method developed in this laboratory (VÁMOS-VIGYÁZÓ *et al.*, 1973). The assay is based upon spectrophotometric optical density (o.d.) determination at 400 nm of the coloured compounds formed from pyrogallol by enzyme action. If not otherwise stated, measurements are carried out on a suspension of peeled comminuted potatoes aerated at pH 6.2 and 30°C for periods of 30 and 60 minutes, respectively, during which interval readings are taken every 10 minutes. Under the experimental conditions changes in optical density are, in the interval of 0 to 60 minutes, in linear relationship with time. Enzyme activity is expressed in terms of rate of change in optical density, as indicated by the slope of the linear regression line calculated from the values corresponding to different incubation intervals. Unit enzyme activity causes a change in o.d. modulus of $1 \cdot 10^{-4}$/min. Activity related to 1 g of potatoes will be, hereafter, referred to as enzyme concentration, with the exception of the experiments on temperature and pH dependence, where this denomination might lead to erroneous conceptions. It follows, though, from the experimental conditions that the numerical values of activity, *i.e.* reaction rate (Δo.d.$_{400}$ min^{-1}) and enzyme concentration (Δo.d.$_{400}$ min^{-1}g^{-1}) differ only by the factor of 5.

Potato suspensions were prepared in triplicate and activity measurements carried out in three parallels on each suspension.

1.3. Temperature and pH

Enzyme activities were determined at temperatures of 25, 30 and 35°C and at pH values of 5.0, 6.2, 6.7 and 7.3, respectively. The buffers used to obtain the required pH values are described in Part I of this paper (VÁMOS-VIGYÁZÓ *et al.*, 1973).

1.4. Storage of potatoes

One lot each of the commercial varieties was stored in the refrigerator at +5°C from the end of October till the beginning of April. Activity was determined at least monthly from samples taken at random.

2. Results

2.1. Dependence of enzyme activity on temperature

The relationship between enzyme activity as related to unit weight of potatoes and temperature of activity measurements is illustrated in Fig. 1.

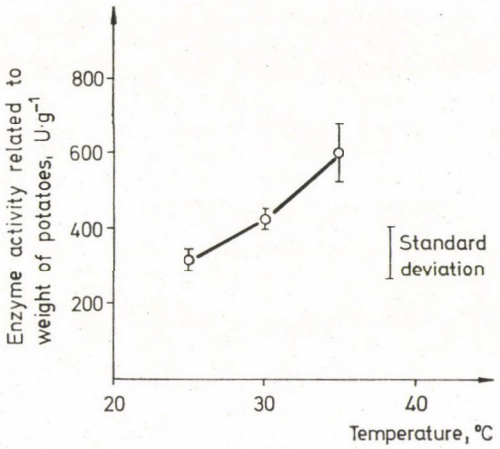

Fig. 1. Changes in the o-diphenol oxidase activity of potatoes with temperature

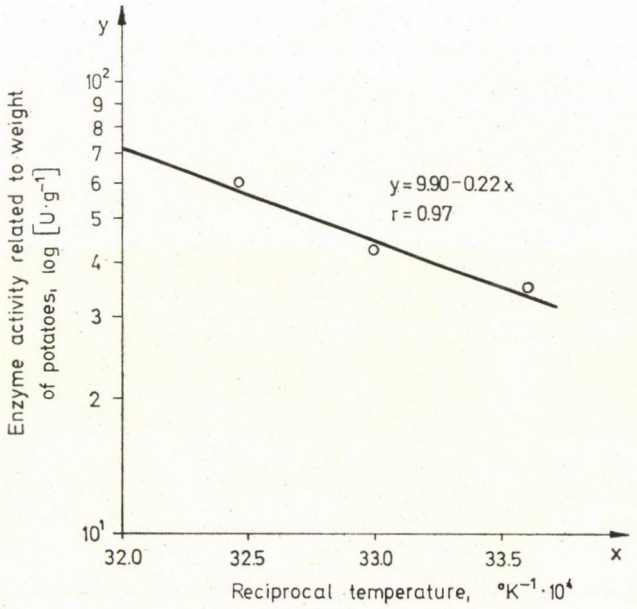

Fig. 2. Relationship between the logarithm of enzyme activity and reciprocal temperature

As can be seen, activity increases with temperature: at 35°C its value is nearly doubled as compared to the value obtained at 25°C. It can be further shown that the changes in activity with temperature follow Arrhenius' equation (Fig. 2): on plotting the activity on a logarithmic scale against reciprocal absolute temperature a straight line was obtained for the range investigated.

2.2. *Dependence of enzyme activity on pH*

The experiments carried out in order to investigate the pH-dependence of potato o-diphenol oxidase activity had been partly published in Part I of this paper (VÁMOS-VIGYÁZÓ *et al.*, 1973) merely to justify the selection of pH 6.2 for the activity measurements. On further inspection of the same experimental

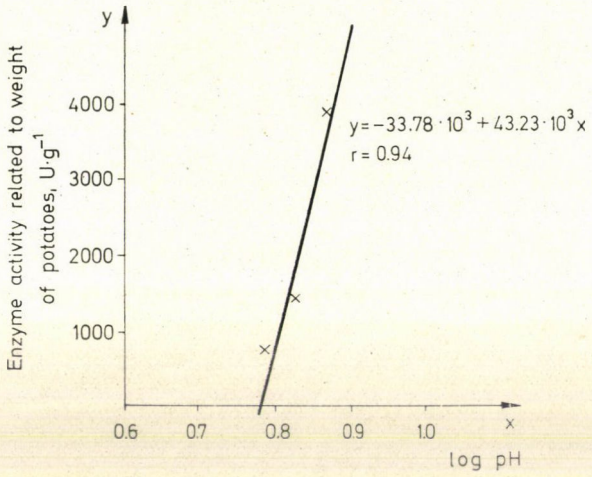

Fig. 3. Relationship between enzyme activity and the logarithm of pH

data (Fig. 8, loc. cit.) an exponential relationship could be established between activity and pH, apart from the value of 0 found at pH 5.0. This is shown in Fig. 3, where activity as related to unit potato weight is plotted against log pH.

The linear regression equation describing the logarithmic plot has been calculated from three points of measurement (*i.e.* the values obtained at pH 6.2, 6.7 and 7.3, respectively), and yields a good correlation.

2.3. *Distribution of the enzyme in the potato tuber*

Enzyme concentration values of an average sample of the peeled tuber, of the parts around the eyes and of the rest of the mesocarp as well as of the peelings are shown in Table 1. The table contains further the weight of these parts, their total enzyme content and the percentage distribution of the latter in the parts of the tuber.

According to the data of the table, enzyme concentration in the peelings and in the tissue around the eyes is — in agreement with general knowledge on the subject — significantly higher than in other parts of the mesocarp. No significant difference could, however, be noted between the enzyme con-

Table 1

Distribution of o-diphenol oxidase in various parts of the potato tuber

Part of the tuber	Weight*			Enzyme concentration*		Total enzyme content	
	g		%	$U \cdot g^{-1}$		U	%
	$\bar{x}$	s		$\bar{x}$	s		
Peelings	13.0	0.8	18.1	730	100	9 490	27.6
Tissue around the "eyes"	2.2	0.6	3.1	550	15	1 210	3.4
Mesocarp without tissue around the "eyes"	56.5	2.6	78.8	425	15	24 012	69.0
Average sample of peeled tubers	—	—	—	450	25	—	—
Total	71.7	—	100.0	—	—	34 712	100.0

*: mean of 4 tubers
U: activity unit
$\bar{x}$: mean
s: standard deviation

Statistical evaluation:

Significance levels of the enzyme concentration values

	Peelings	Around eyes	Mesocarp without eyes
Around eyes	***		
Mesocarp without eyes	***	***	
Peeled average	***	***	0

0 = no significant difference ($P > 0.05$)
*** = very highly significant difference ($P < 0.01$)

centration values of the latter and of the average sample of the peeled potato. As can be seen from the percentage distribution of total enzyme activity, only a negligible part of it (about 4%) is present in the tissue of higher enzyme concentration around the eyes.

2.4. Changes in enzyme concentration during storage

Changes in enzyme concentration during a 5-month cold storage period of 3 potato varieties are represented in Fig. 4.

It can be seen that the differences in enzyme concentrations noted at the beginning of the storage period (in November and December) between the 3 varieties decreased by the 60th or 70th day. From this time on, enzyme

concentrations vary only to a lesser degree, although the variations are significant. This is, however, probably due to differences in the enzyme content of the individual tubers, *i.e.* inhomogeneity of the experimental material. (Attempts were made at minimizing this effect by preparing the potato suspensions from average samples of 3 tubers.)

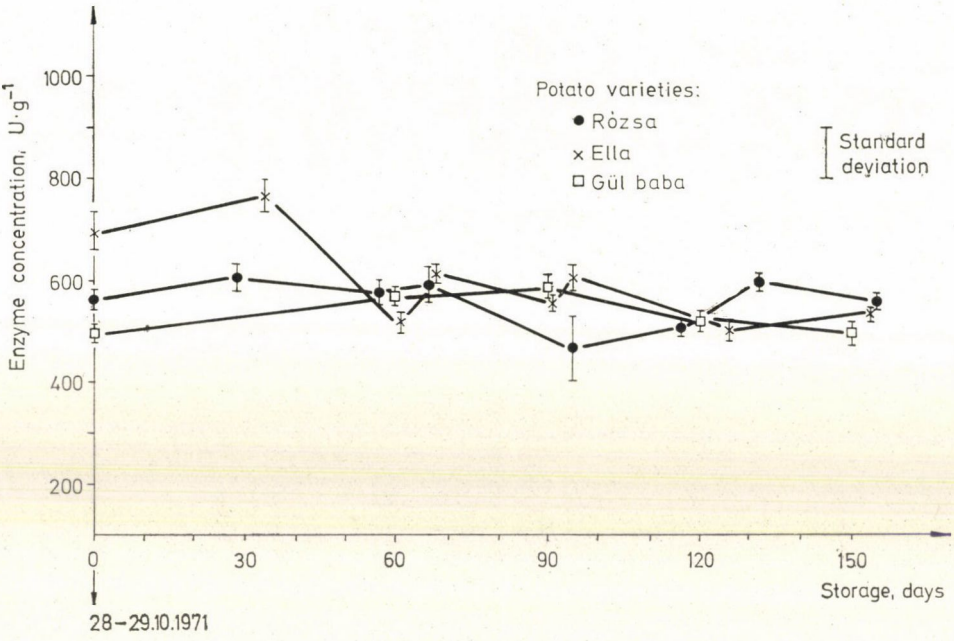

Fig. 4. Changes in o-diphenol oxidase content of different potato varieties during storage

2.5. Enzyme content of experimental potato varieties

Enzyme concentrations of the eight experimental potato varieties obtained in November 1971 by courtesy of the Development Laboratory of the Hungarian Enterprise of the Refrigerating Industry are represented in Fig. 5.

It seems a striking feature of the results that the enzyme concentrations in samples No. 1 and 3, 2 and 4, 5 and 8 as well as 6 and 7, respectively, are in numerical agreement. As shown by analysis of variance, significant differences could be found only between the lowest and highest values of enzyme concentration, present in the sample pairs No. 2 and 4 and No. 5 and 8, respectively.

As can be seen from Fig. 6, the lowest enzyme concentration in the experimental samples is notably lower than the values established for the commercial varieties investigated: o-diphenol oxidase content of the experimental

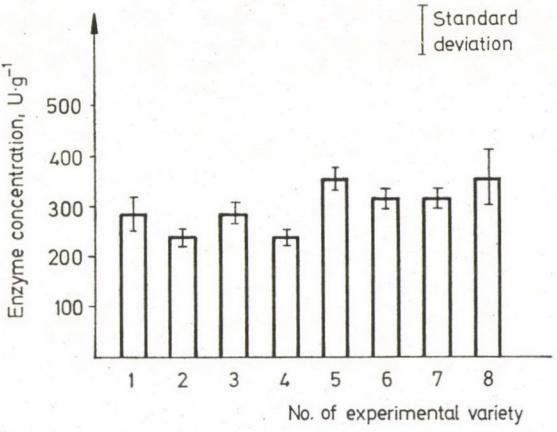

Fig. 5. o-Diphenol oxidase content of experimental potato varieties

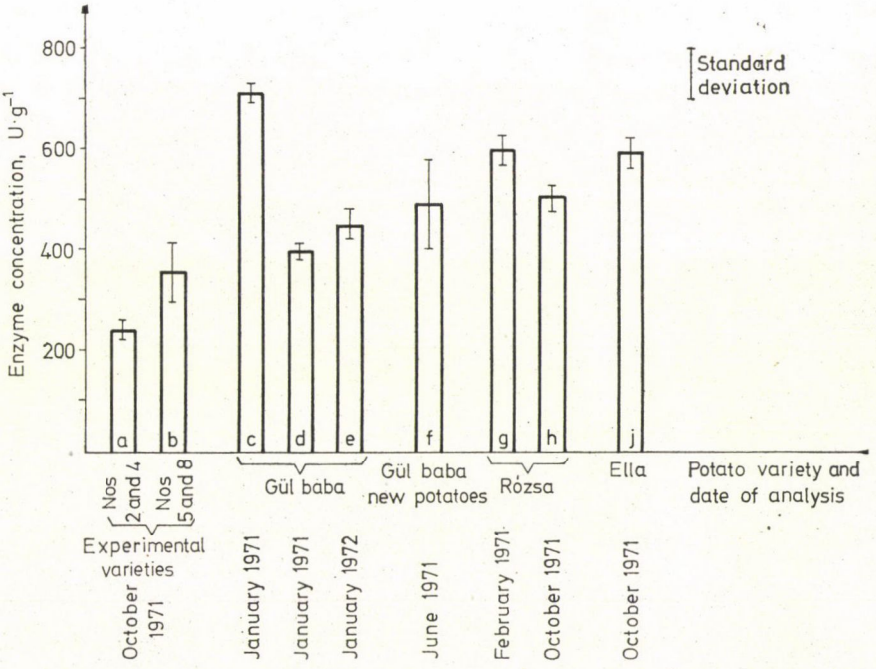

Fig. 6. Comparison of the enzyme content of the potato lots investigated

samples No. 2 and 4 was about 1/3 of the highest and about 60% of the lowest value found in the samples of the variety Gül baba.

The figure shows further that there are great differences in o-diphenol oxidase content between the batches investigated of the same variety.

3. Conclusions

Some of the results obtained — especially those concerning temperature and pH dependence of the enzyme and its distribution in different parts of the potato tuber — amply justify the experimental conditions chosen for activity measurements as described in Part I.

The temperature dependence of enzyme activity (Figs. 1, 2) shows that no inactivation occurs around the temperature chosen for activity determination, moreover, the validity of the Arrhenius equation allows to extrapolate activity values established at 30°C to lower or higher temperatures, at least in the range investigated. The increase in activity to its 1.76-fold by raising the temperature from 25 to 35°C is in good agreement with theoretical considerations assuming an activation energy value of 10 000 cal and yielding 1.73 for the given range on rough calculation as suggested by GUTFREUND (1965). However, taking into account the heat sensitivity of enzyme proteins, experiments should be extended to a wider range of temperature in order to ensure reliable data for the processing industries.

The selection of pH 6.2 for activity determinations had been, as explained in Part I of this paper, primarily motivated by the aim at carrying out measurements near the own pH of potatoes. According to the exponential relationship between pH and activity (Fig. 3) the value of the latter would reach zero around pH 6 (at log pH = 0.78142), *i.e.* in close proximity to the pH chosen for activity determinations. It may, however, be assumed that the exponential relationship does not hold for the pH range between 5.0 and 6.2. Investigation into this problem would be the more interesting as data contradictory to these findings were reported in the literature: ALBERGHINA (1964) as well as PATIL and ZUCKER (1965) found for a purified potato o-diphenol oxidase preparation an activity maximum at pH 5 on chlorogenic acid substrate and a decrease in activity with increasing pH up to the value of 7, where a shoulder in the activity curve could be observed, followed by a steep drop towards the alkaline region. These authors attribute the behaviour of the enzyme in the neutral range to ionization of the substrate, which being different from the one used in the present study, would account for the discrepancies in the results. It must be noted that, on the other hand, the similarity of the results of activity measurements — at a pH not specified — on chlorogenic acid and pyrogallol was stressed especially for potato extracts by SCHWERDTFEGER (1970). The activity maximum at pH 5 of the purified enzyme is explained by the ionization of histidine residues as part of the active site in the protein molecule (PATIL & ZUCKER, 1965). This, of course, cannot be expected to occur in a similar way in cell-bound enzymes as dealt with in the present study. The exponential increase of *in situ* enzyme activity with increasing pH has lately been corroborated by similar findings obtained for peaches (VÁMOS & KISS, 1972).

The data in Table 1 confirm that the enzyme content of the parts to be processed may be duly represented by using an average sample of peeled potatoes. Minor deviations from these data may, however, occur, since hand peeling was used throughout the experiments.

The results of the winter storage experiments obtained so far do not allow any conclusion of importance as to the behaviour of the enzyme in the three varieties investigated or as to changes of potato o-diphenol oxidase in general. It is, perhaps, interesting that at the end of the storage period, although sprouting could not be prevented in spite of low temperature and darkness, only the variety Rózsa showed an increase in enzyme concentration without, however, exceeding the initial value (Fig. 4). Since but slight variations in enzyme concentration were observed during winter storage, this problem will be considered in future work as of secondary importance, although changes occurring simultaneously in polyphenol content or in texture of the potato may lead to a different browning tendency even if the o-diphenol oxidase content remains unchanged.

From the practical point of view the low enzyme concentration of the experimental varieties as compared to that of the commercial ones may be considered a most important result (Fig. 5). Conclusions as to browning tendency in processing cannot, however, be drawn without the knowledge of the amounts of substrate (polyphenols) available in the tissues.

Thus, further work will be focussed upon the investigation into the browning tendency of new or experimental varieties and the simultaneous study of their o-diphenol oxidase and polyphenol content.

*

The authors are indebted to the Development Laboratory of the Hungarian Enterprise of the Refrigerating Industry for the supply of experimental potato varieties.

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EFFECT OF IRRADIATION ON THE PROPERTIES OF SOME SHRINKING POLYMER FILMS

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Shrinking polymer films (polyethylene, polyvinylidene chloride, polyester) suitable for use in the food industry were studied with the intention to determine the effect of radurizing doses (800 krad and below) on changes in the proportion of crystalline parts in the polymer, and on the tensile strength, elongation at break and shrinkage of the film.

Changes in the crystalline/amorphous ratio in the polymer were determined by means of infra-red spectrophotometry (Figs. 1, 3 and 5). Calculations based on spectral data showed no significant changes in the ratio of crystalline fraction of any of the films, as a function of radurizing doses (Figs. 2, 4 and 6).

Tensile strength and elongation at break tests were carried out by means of standardized instruments and methods. It was found that the tensile strength of the polyethylene film decreased by about 25% as an effect of irradiation (Fig. 7), while the same treatment caused no significant changes in the elongation at break (Fig. 10). The tensile strength of the polyvinylidene chloride film suffered a decrease of roughly 15% (Fig. 8), its elongation at break an about 30% (Fig. 11) decrease when irradiated. Radiation treatment caused a decrease of less than 10% in tensile strength of the polyester film (Fig. 9) and a more than 10% change in elongation at break (Fig. 12).

Shrinkage was tested by the method of the author which is based on the practical application of the film. The tests indicated no significant changes in the shrinkage of radiation treated polymers.

The results of the tests led to the conclusion that radurizing doses caused no such change which would affect the applicability of polymer films to the wrapping and packaging of foods subjected to irradiation or would make the films unsuitable for the protection of the goods.

Changes due to the irradiation of polymers used in the food industry were studied primarily from the aspect to improve some property — usually the mechanical strength — of the polymer by means of gamma radiation, whereby the polymer might become more suitable for use in this industry.

However, the doses capable of influencing the properties of polymers are higher by 1 or 2, or sometimes even 3 orders of magnitude than those used for the preservation of food. This explains the fact that the attention of researchers engaged in the modification of polymers has been centered on doses considerably higher than the krad range and this has been the subject of many theoretical and experimental studies.

Investigation of the applicability of ionizing radiation in the food industry necessitated, however, the study of the effect of radurizing or "pasteurizing"

radiation (in the 10^2 krad range) on polymers used for the packaging of foodstuffs.

The aim of this work was to detect changes in the structure and properties of polymers particularly suitable for packaging occurring on exposure of these poly mers to doses of the order of some krad and to establish whether such changes would affect their applicability in direct contact with various foodstuffs when a very high standard of hygiene should be observed.

The tests to be described below aimed to ascertain any significant change in the properties of shrinking polymer films, particularly of low density polyethylene, polyvinylidene chloride and polyethylene terephthalate films when exposed to gamma-ray irradiation of the order of less than 1 Mrad.

1. Materials and methods

1.1. Materials

The shrinking films used in the tests were suitable, both from the technological and economic aspect, for the protective wrapping of foodstuffs and, last but not least, conformed to the demands of food hygiene.

1.1.1. Polyethylene film. The low density (0.91 g/cm^3), 0.040 mm thick polyethylene film used in the test was produced and made mechanically suitable for shrinking by the Tiszai Vegyi Kombinát, Hungary

1.1.2. Polyvinylidene chloride film. The 0.03 mm thick shrinking polyvinylidene chloride film tested was a product of the Firm Åkerlund and Rausing.

1.1.3. Polyester film. Of the various polyester films a shrinking 0.020 mm thick polyethylene terephthalate film of the firm Kalle AG was tested.

1.2. Methods

1.2.1. Irradiation. For the irradiation of the films the ^{60}Co gamma radiation source of 50 000 Ci nominal strength of the Central Food Research Institute was used. The temperature of irradiation was 10—15°C. The dose rate was 100 to 200 krad per hour at the site of irradiation. The adsorbed dose was determined by chemical dosimetry using the method of WEISS *et al.* (1955).

1.2.2. Infra-red spectrophotometry. Specimens to fit the instrument were cut from samples which had been irradiated with 100, 200, 400 and 800 krad, resp., the surface of the specimens carefully cleaned and their spectra taken with the UNICAM type SP 200 recording spectrophotometer at 1/2 E energy level, room temperature and normal humidity. The spectra were taken against air with 5 samples at each dose level.

1.2.3. Tensile strength and elongation at break. The tests were carried out with the FMPw type 500 tensile strength tester of the VEB Werkstoff Prüfmaschinen according to Hungarian standards MSz 5360—52 and MSz 5355—51,

The measuring limit was adjusted to 100 kp, the read-off accuracy of tensile strength tests was 0.2 kp, that of the elongation at break tests was 0.5%.

From the films irradiated with 10, 20, 40, 100, 200, 400 and 800 krad, resp., strips of 30 mm width and 200 mm length in machine and transverse direction were cut. 10 samples were taken at each dose level.

1.2.4. Shrinkage. From the films which had been irradiated with 100, 200, 400 and 800 krad, resp., five 100 mm long and 30 mm wide samples were cut per dose level and immersed in 90 ± 1°C water for 15 seconds. Changes in dimensions were measured with the aid of a caliper with an accuracy of 0.1 mm.

1.2.5. Statistical evaluation of results. The experimental data obtained were evaluated by means of mathematical statistical methods (SVÁB, 1967). The conspicuously deviating values were checked by means of Dixon's r criterion and those found outstanding were excluded from the calculations. The homogeneity of deviations was checked by means of Bartlett's test and analysis of variance was applied to the study of the effect of ionizing radiation as a function of the changes in the properties of the polymer films. The experimental data of the irradiated films were compared to those of the untreated samples using Student's t test.

2. Results

2.1. Evaluation of the data of infra-red spectroscopy

Ample literature is available on the evaluation of infra-red spectroscopic data of various polymers and on the interpretation of the intensity changes of single bands. Due to lack of space it is not possible to deal here in detail with the various aspects of infra-red spectroscopy, so that we shall restrict ourselves to the discussion of the significant changes in the intensity of the characteristic bands and to the ratio of crystalline to amorphous parts, as those most characteristic of the polymer.

2.1.1. Polyethylene film. The infra-red absorption spectra of untreated shrinking, low density, polyethylene films and of those treated with 800 krad are shown in Fig. 1.

The ratio of the crystalline portion in the structure of the polyethylene film was determined from the change in the intensity ratio of the absorption bands at 1299 cm^{-1} and 1887 cm^{-1}, as recommended by HENDUS and SCHNELL (1961). The experimental results and the results of calculations are shown in Fig. 2.

2.1.2. Polyvinylidene chloride film. The infra-red absorption spectra of untreated polyvinylidene chloride films and of those treated with 800 krad are presented in Fig. 3.

The effect of irradiation on the structure of the polyvinylidene chloride film was determined from the change of the vinylidene chloride to vinyl

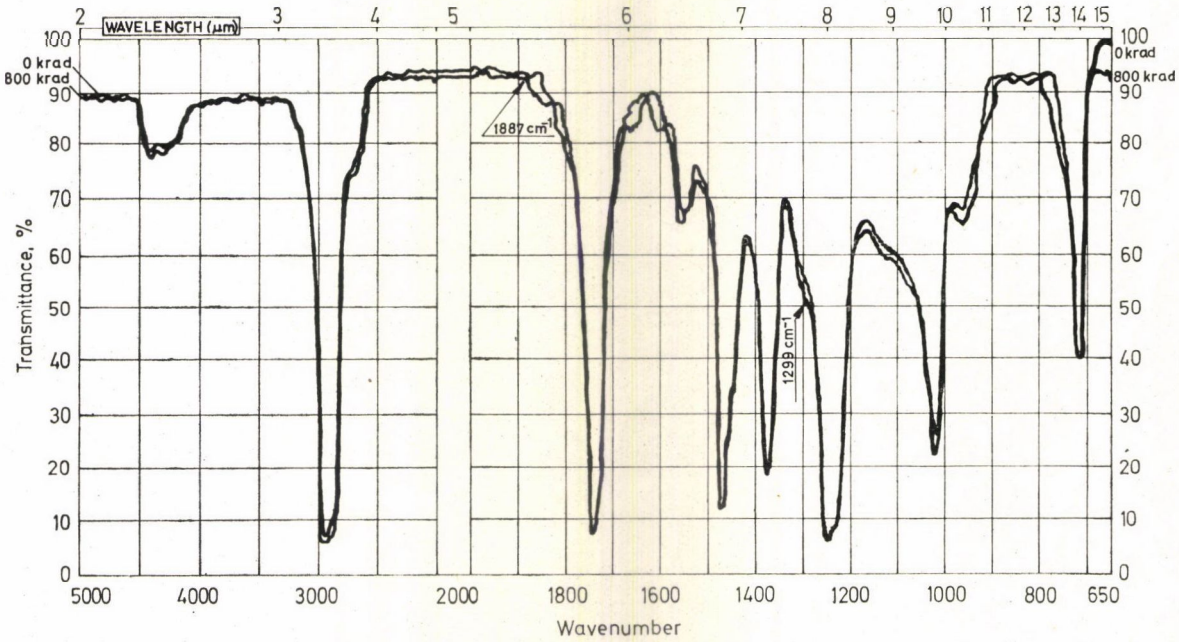

Fig. 1. IR absorption spectra of untreated and irradiated (800 krad), 0.040 mm thick shrinking polyethylene films

chloride ratio by means of the method recommended by NARITA *et al.* (1959). This involves the comparison of the quotient of the absorption values pertaining to 1206 cm^{-1} and 1355 cm^{-1} wavenumbers in the untreated and radiation treated films. The experimental data were subjected to statistical evaluation, as described in para. 1.2.5. The results are illustrated in Fig. 4.

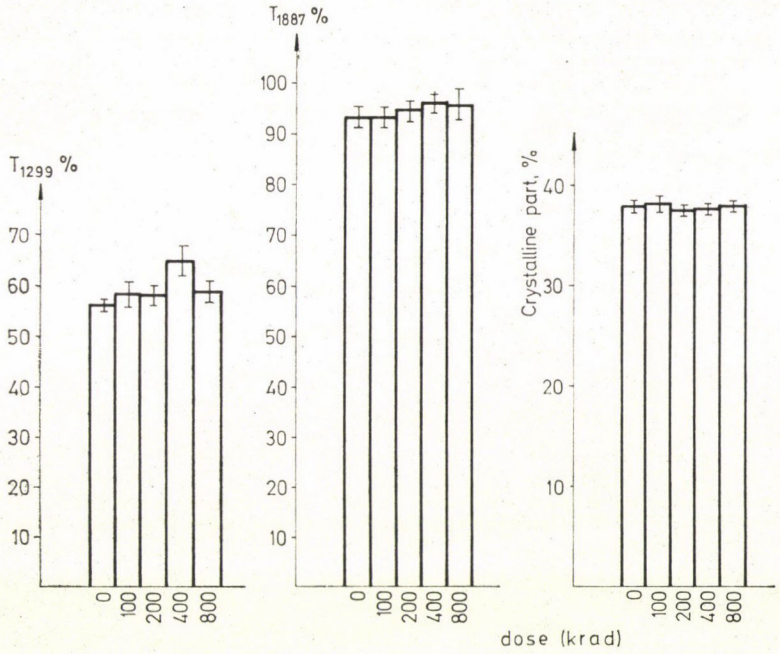

Fig. 2. Comparison of the amount of the crystalline part, and of the transmittance (from which the crystalline part was determined) of untreated and radiation treated 0.040 mm thick, low density, shrinking polyethylene films in the infra-red region. Transmittance was measured at 1299 cm^{-1} and 1887 cm^{-1} (T_{1299} and T_{1887}). The heights of the columns indicate the means of 5 measurements, the vertical bars stand for the double of the standard deviations

2.1.3. Polyester film. The infra-red absorption spectra of untreated polyethylene terephthalate type polyester film and of those treated with 800 krad are given in Fig. 5.

The changes caused by irradiation in the polyester film were calculated from the ratio of the intensities of bands at 972 cm^{-1} and 795 cm^{-1} (COBBS & BURTON, 1953; THOMPSON & WOODS, 1955; WARD, 1956). In this way the change in the proportion of the amorphous to the crystalline part was obtained from the relationship between transmittance and specific gravity which indicates the amount of the amorphous content. The experimental results and the results of calculations are given in Fig. 6.

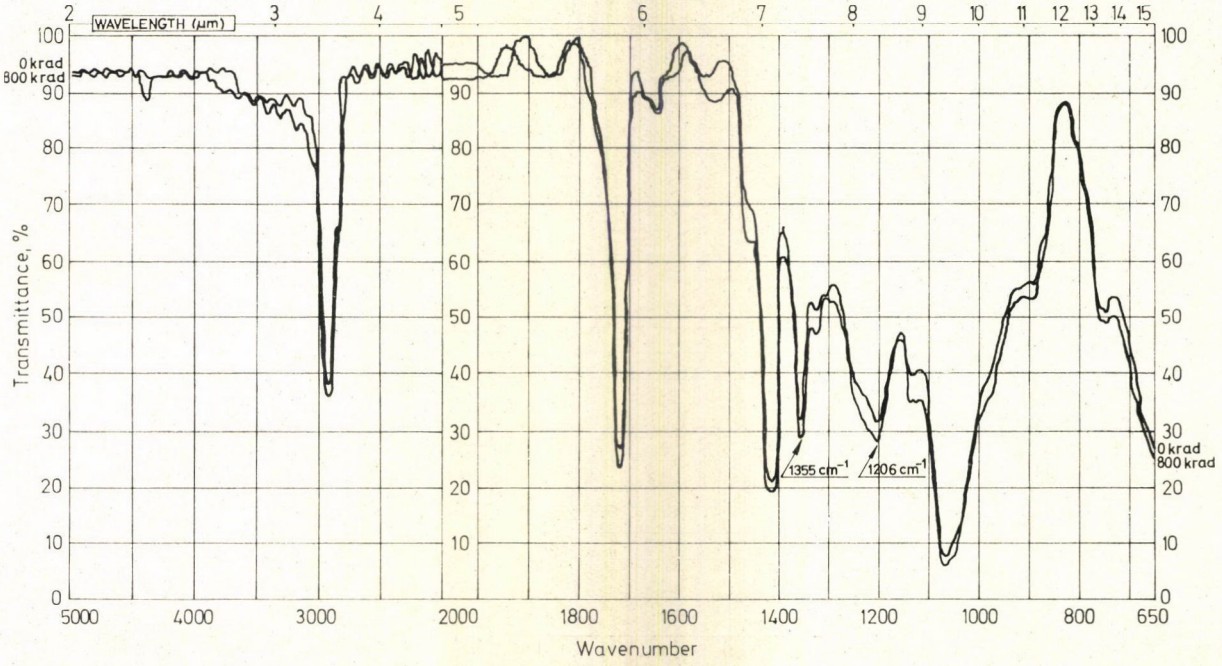

Fig. 3. IR absorption spectra of untreated and irradiated (800 krad) polyvinylidene chloride films

2.2. The effect of ionizing radiation on tensile strength and on elongation at break

The tensile strengths of untreated films and of films irradiated with 10, 20, 40, 100, 200, 400 and 800 krad, respectively, were measured both in machine and in transverse direction. The results obtained on polyethylene films are shown in Fig. 7, those of polyvinylidene chloride films and polyester films in Figs. 8 and 9, resp.

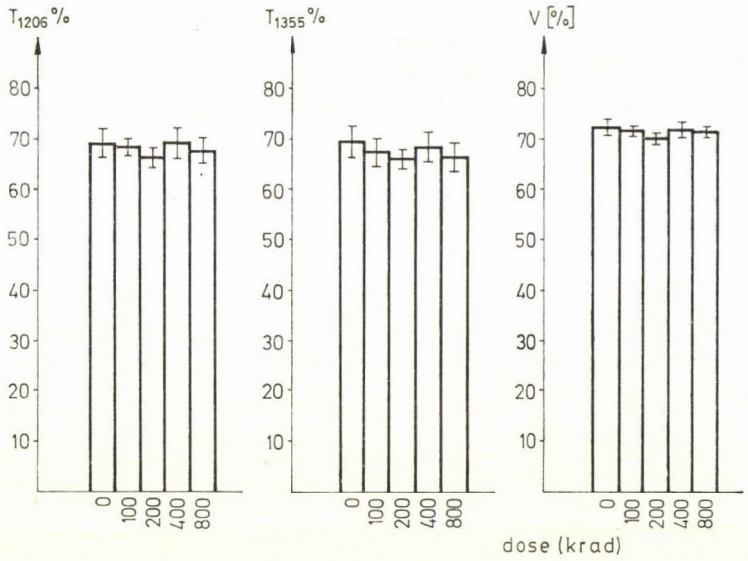

Fig. 4. Comparison of the vinylidene chloride contents (V %) and of the transmittances (from which the vinylidene content is determined) of untreated and radiation treated, 0.030 mm thick, shrinking polyvinylidene chloride films, in the infra-red region. Transmittance was measured at 1206 and 1355 cm^{-1} (T_{1206} and T_{1355}). The heights of the columns represent the means of 5 measurements, the vertical bars stand for the double of the standard deviations

The results of elongation at break tests for polyethyiene films are presented in Fig. 10, those for polyvinylidene chloride and for polyester films in Figs. 11 and 12, resp.

2.3. The effect of ionizing radiation on shrinkage

Figs. 13 sums up the results of shrinkage tests in machine and transverse direction. The samples were: untreated polyethylene, polyvinylidene chloride and polyester films and the same films irradiated with 100, 200, 400 and 800 krad, resp.

The results obtained with radiation treated films were compared to those of untreated films by means of Student's t test and in none of the cases could a significant difference between the results be detected.

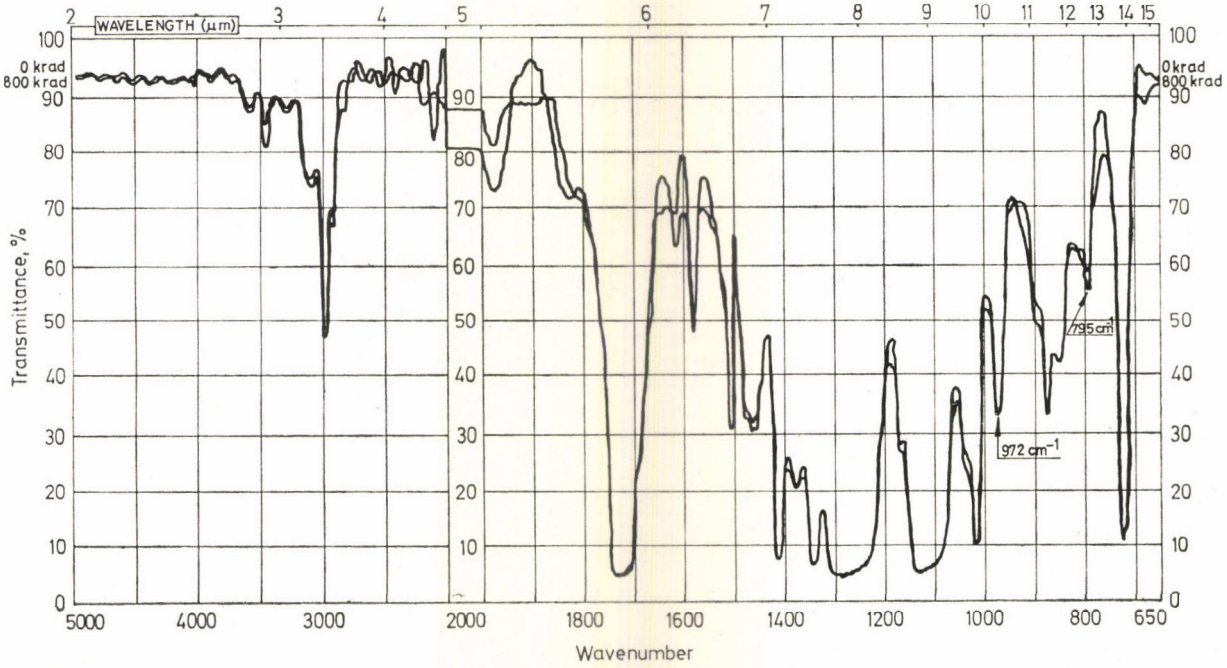

Fig. 5. IR absorption spectra of untreated and irradiated (800 krad), 0.020 mm thick polyester films

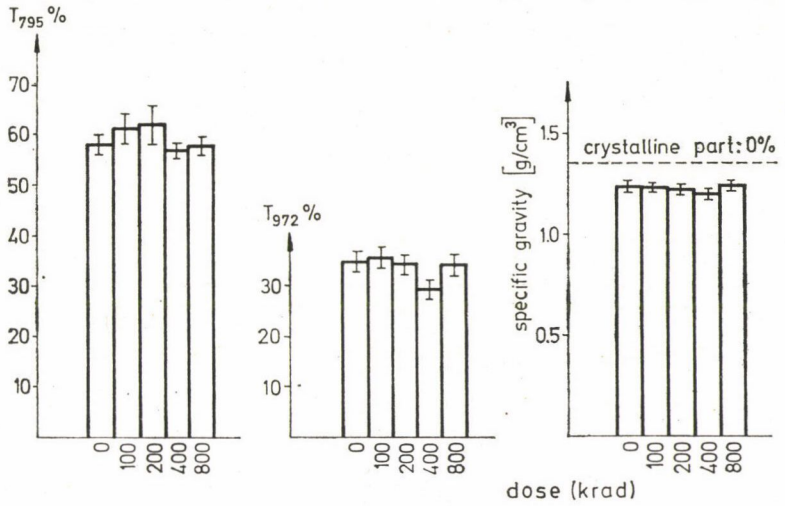

Fig. 6. Comparison of the specific gravity, and of the transmittance (from which specific gravity is determined) of untreated and radiation treated, 0.020 mm thick, shrinking polyester films in the infra-red region, at 795 and 972 cm^{-1} (T_{795} and T_{972}). The heights of the columns indicate the means of 5 measurements, the vertical bars stand for the double of the standard deviations

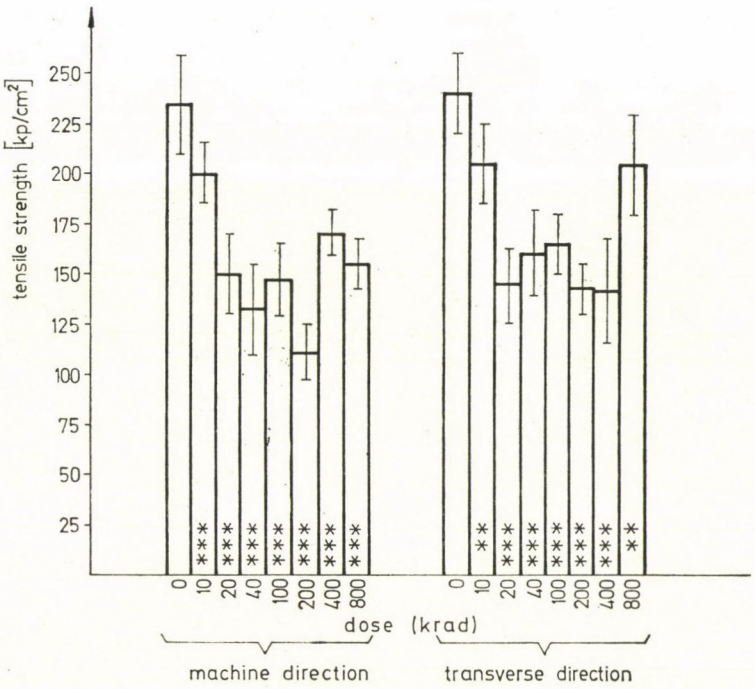

Fig. 7. Comparison of the tensile strength both in machine and transverse direction of untreated and irradiated, 0.040 mm thick, low density shrinking polyethylene films. The heights of the columns indicate the means of 10 measurements, the vertical bars stand for the double of the standard deviations. **: highly significant difference at the probability level P = 1%; ***: very highly significant difference at the probability level P = 0.1%

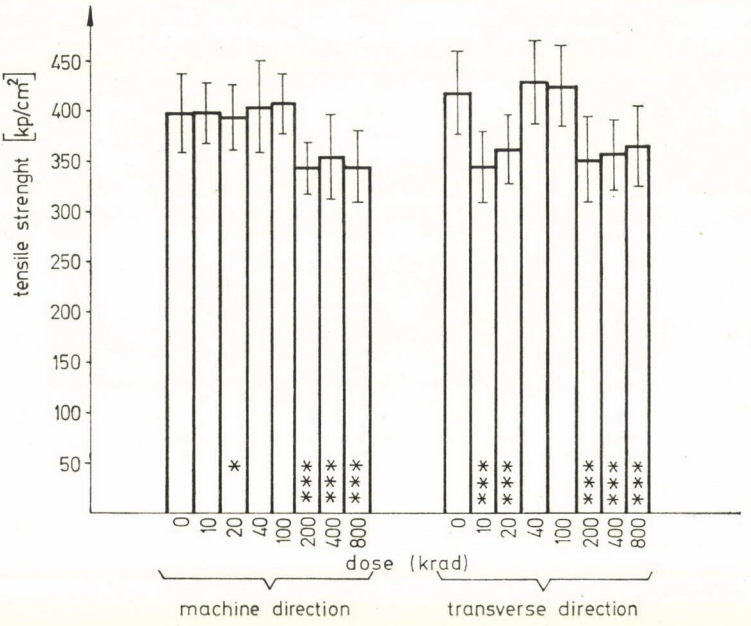

Fig. 8. Comparison of tensile strength values both in machine and transverse direction of untreated and irradiated, 0.030 mm thick, shrinking polyvinylidene chloride films. The heights of the columns represent the means of 10 measurements, the vertical bars stand for the double of the standard deviation. *: significant difference at the probability level P = 5%; **: highly significant difference at the probability level P = 1%; ***: very highly significant difference at the probability level P = 0.1%

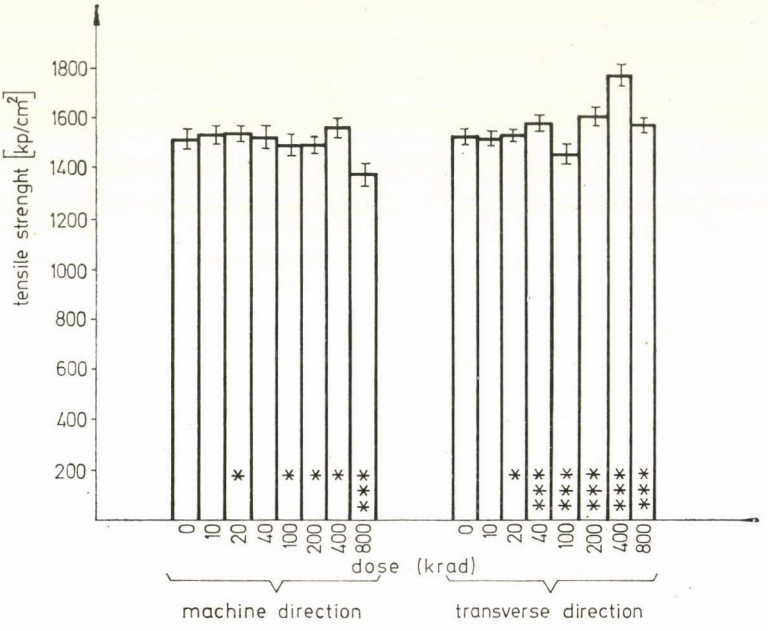

Fig. 9. Comparison of tensile strength values, both in machine and transverse direction, of untreated and irradiated, 0.020 mm thick shrinking polyester films. The heights of the columns represent the means of 10 measurements, the vertical bars stand for the double of the standard deviations. *: significant difference at the probability level P = 5%; ***: very highly significant difference at the probability level P = 0.1%

3. Conclusions

IR spectroscopic structure research intended to reveal changes in the crystalline to amorphous ratio in polyethylene (Fig. 2) and polyester (Fig. 6) films, as well as changes in the ratio of vinyl chloride to vinylidene chloride in

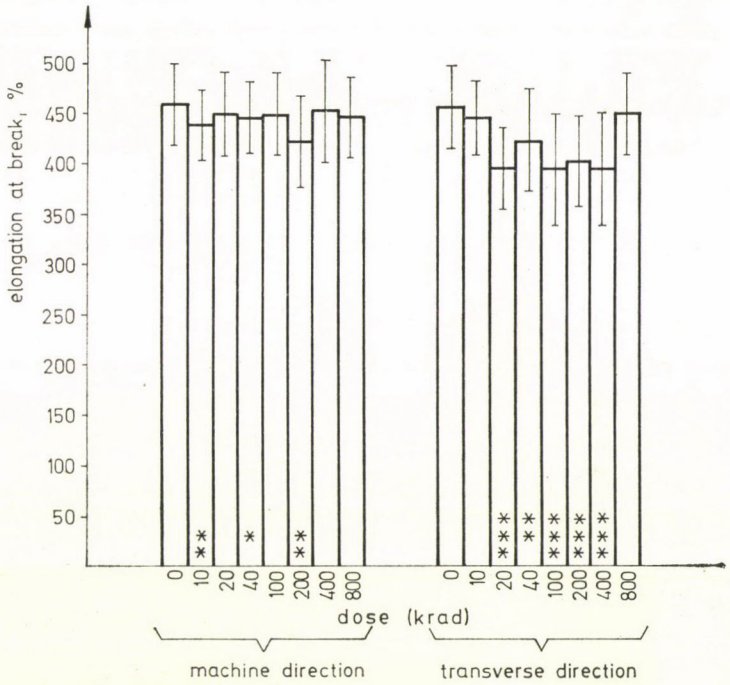

Fig. 10. Comparison of elongation at break values, in both machine and transverse direction, of untreated and irradiated, 0.040 mm thick, low density, shrinking polyethylene films. The heights of the columns represent the mean values of 10 measurements, the vertical bars stand for the double of the standard deviations. *: significant difference at the probability level P = 5%; **: highly significant difference at the probability level P = 1%; ***: very highly significant difference at the probability level P = 0.1%

the polyvinylidene film (Fig. 4) indicated no significant difference between the radiation treated and untreated films.

The tensile strength of the polyethylene film suffered a loss of about 25% both in the machine and in the transverse direction (Fig. 7), but no unequivocal relationship was found between this value and the radiation dose. Analysis of variance of elongation at break values gave no significant difference between the mean values (Fig. 10).

Doses of 200, 400 and 800 krad caused a loss of roughly 15% in the tensile strength of the polyvinylidene chloride film in machine direction. In transverse

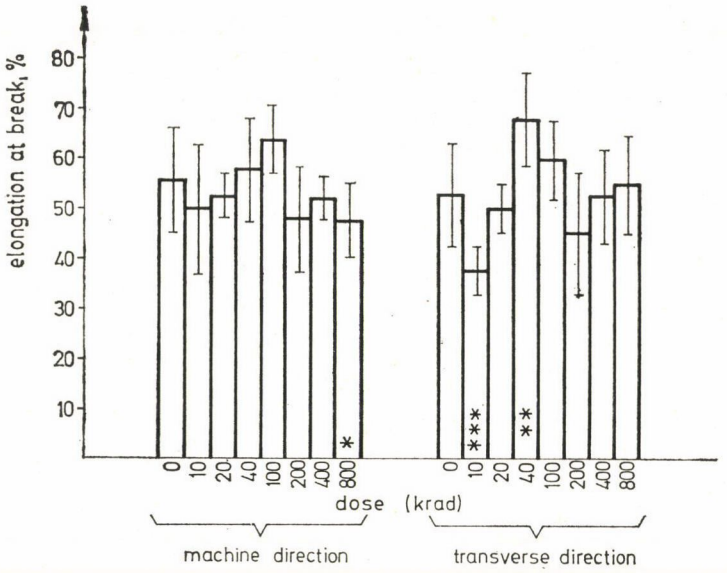

Fig. 11. Comparison of the elongation at break, in both machine and transverse direction, of untreated and irradiated, 0.030 mm thick, shrinking polyvinylidene chloride films. The heights of the columns represent the mean values of 10 measurements, the vertical bars stand for the double of the standard deviations. *: significant difference at the probability level P = 5%; **: highly significant difference at the probability level P = 1%; ***: very highly significant difference at the probability level P = 0.1%

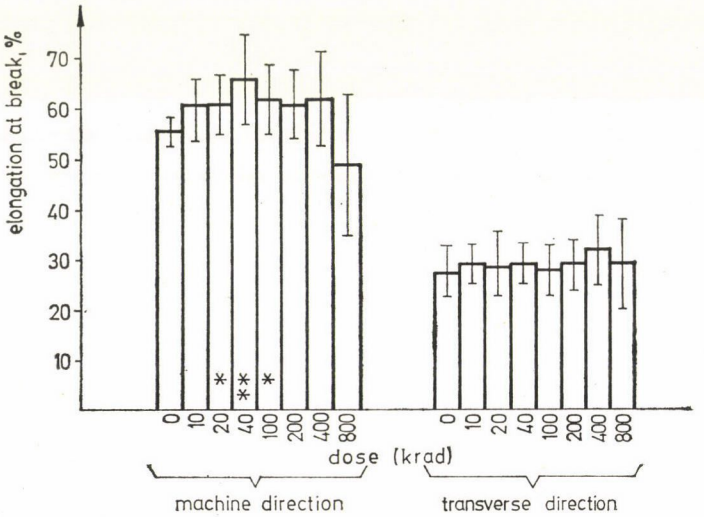

Fig. 12. Comparison of elongation at break values, in both machine and transverse direction, of untreated and irradiated, 0.020 mm thick shrinking polyester films. The heights of the columns represent the mean values of 10 measurements, the vertical bars stand for the double of the standard deviations. *: significant difference at the probability level P = 5%; **: highly significant difference at the probability level P = 1%

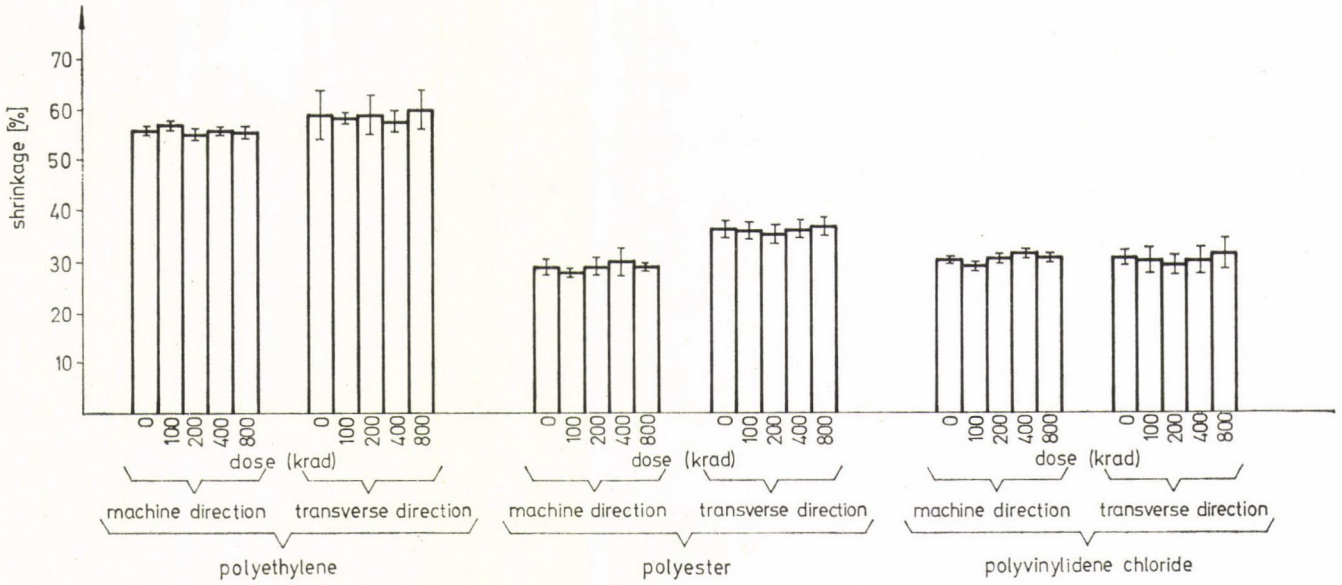

Fig. 13. Comparison of the shrinkage of untreated and irradiated, 0.040 mm thick, low-density polyethylene, 0.030 mm thick, polyvinylidene chloride and 0.020 mm thick polyester films, in machine and transverse direction. The heights of the columns represent the means of 5 measurements, the vertical bars stand for the double of the standard deviations

direction doses of 10, 20, 200, 400 and 800 krad brought about an almost equal decrease in tensile strength (Fig. 8).

The average decrease of elongation at break values, both in machine and in transverse direction was the same — about 35% — in all radiation treated samples (Fig. 11).

Statistical evaluation of the results of tensile strength tests on polyester films (Fig. 9) showed a significant difference of about +4% and −10% between the untreated and irradiated samples both in the machine and transverse direction.

In the machine direction a significant difference of 10 to 15% was found in the elongation at break value of the untreated samples and of those irradiated with 20, 40 and 100 krad, resp., (Fig. 12), but no such significant difference was observed in the mean values of the transverse elongation at break of the same samples.

Investigation of shrinkage as a function of the radiation dose (Fig. 13) has revealed that ionization radiation causes no significant change in this property of the polymers.

Summing up the results we may conclude that radurizing gamma-ray radiation doses caused no such change in the films under investigation which would make these films unsuitable for use in the food industry.

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INVESTIGATIONS INTO THE PRODUCTION OF MILK CLOTTING ENZYME PREPARATIONS

PART I. — ENZYME PRODUCING CAPACITY OF *MUCOR PUSILLUS* LINDT IN SURFACE CULTURE

K. POZSÁR-HAJNAL, L. VÁMOS-VIGYÁZÓ and E. HEGEDÜS-VÖLGYESI

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Based on relevant literature the milk clotting enzyme producing capacity of the *Mucor pusillus* Lindt strain was investigated. Experiments were carried out in surface cultures on moistened bran medium.

In surface cultures on bran a 5-day incubation period at 35°C is favourable for the biosynthesis of the enzyme, both in flasks and trays. An about ten-fold air exchange per hour is required by the culture in covered trays.

The crude preparation obtained by extracting the comminuted bran culture at the end of the fermentation period with 5 volumes of water as related to bran weight and precipitating the extract thus obtained with 3 volumes of methanol, followed by centrifuging, filtration and drying had a rennin concentration of 20 000—60 000 SU g^{-1} (SU = Soxhlet Unit). The ratio of milk clotting and proteolytic activity was 10—17.

In the last four years experiments were carried out at the Central Food Research Institute to replace calf rennet in cheese manufacture by enzymes of microbial origin. As reported earlier (VÁMOS *et al.*, 1968a, 1968b) this subject was investigated in many countries. The consultation, organized by FAO in 1968 in Rome, shows the general interest in this field (ANON, 1968). However, it appears from the related literature (MAASS, 1967; SRINIVASAN *et al.*, 1962; CHEBOTAREV *et al.*, 1966; BARKAN *et al.*, 1964; PUHAN, 1966; SCHULZ *et al.*, 1967; BEHNKE, 1968; SUZUKI *et al.*, 1968; KIKUCHI *et al.*, 1968a, b) that the various enzyme preparations used are not equally suitable to replace calf rennet in the manufacture of different kinds of cheese. The same observation was made by PULAY (1969) who found that the partial replacement of calf rennet with pepsin was very successful in many kinds of cheese, but could not be applied in the manufacture of Gruyere cheese.

As seen from the above neither of the enzyme preparations of animal, vegetable or microbial origin was found suitable so far to universally replace calf rennet. Thus it was found expedient to launch investigations at the same time along several lines. At first microbial enzyme preparations available on the world market, or under trial in socialist countries and produced on pilot plant scale were subjected to investigation (VÁMOS *et al.*, 1969a, b; VÁMOS & SZEKERES, 1969; VÁMOS & MORVAI, 1969; POZSÁR *et al.*, 1969; KISS, 1969a). Based on the results of these experiments the two preparations most resembling

calf rennet, Meito Rennet prepared of *Mucor pusillus* Lindt in Japan, and Sure Curd prepared of *Endothia parasitica* in the USA were applied in experimental manufacture of soft, semi-hard, hard and white cheese by another research-group of this laboratory (KISS, 1969b).

Most of the microbial rennin preparations on the world market are of fungal origin (ANON, 1967a, b; ANON, 1969; ARIMA & IWASAKI, 1964, 1965; ARIMA *et al.*, 1967; IWASAKI *et al.*, 1967; SARDINAS & FERRY, 1966; SARDINAS, 1968; EDELSTEN *et al.*, 1969; PRINS & NIELSEN, 1970; AUNSTRUP, 1970; KAWAI & MUKAI, 1970; KAWAI, 1970). In agreement with international experiences and because the results of preliminary experiments with bacterial enzymes were not unambiguous it was decided to focus further work on milk clotting enzymes obtained by fermentation with some mould strains.

In this paper the results of experiments carried out with a *Mucor pusillus* Lindt mould strain, obtained from a culture received from Centraalbureau voor Schimmelcultures, Baarn, Holland, are discussed.

1. Materials and methods

1.1. Maintenance of the strain

The *Mucor pusillus* Lindt strain was maintained in test tubes on malt-agar slant of 5% refractometer value. On this medium the mould forms cotton-wool like greyish-white colonies. The microscopic study of micelia shows thick, branched, not septated filaments, sporangiophores with spore-containing sporangia.

The spore suspension obtained by washing the 7-day agar slant with physiological salt solution was used as inoculum.

1.2. Nutrient medium and conditions of fermentation

20 g wheat bran was weighed into 250 ml Erlenmeyer flasks each, 14 ml tap water was added and mixed and sterilized at 133°C for an hour. After cooling each flask was inoculated with 5 ml spore-suspension ($3-5 \cdot 10^{10}$ spores/100 g bran).

Cultivation was continued for 3, 4, 5, 6 or 7 days at 25, 30, 37 and 45°C, respectively. Every experiment was carried out in 8 replicates.

1.3. Extraction of the enzyme

After cultivation the enzyme was extracted from the comminuted bran culture with 100 ml water per flask, containing 0.2 ml toluene. The medium-

water mixture was allowed to stand for 24 hours at room-temperature. The extract was separated from the slurry by centrifuging in a household centrifuge at 1 400 rpm for 5 minutes. The basket of the centrifuge was lined with four layers of thin muslin mull. The liquid phase was again centrifuged on a refrigerated centrifuge at 3 500 rpm for 15 minutes. The enzyme was precipitated in the supernatant with 3 volumes of methanol. It was kept at room temperature overnight, then decanted and the precipitate condensed by centrifuging. It was then suspended in absolute ethanol, filtered through a Buchner funnel layered with asbestos and one layer of filter paper and airdried by suction, till it came off the filter paper easily. Subsequently it was allowed to dry in a thin layer at room temperature. Finally it was ground in a coffee mill and sieved through a silk sieve of 0.16 mm mesh.

1.4. Influence of additives

The following additives were used: 14 ml rehydrated milk to 20 g bran per flask, instead of water, or 100 ml rehydrated milk to 10 g bran, a mixture of superphosphate and ammonium sulphate, or 0.2—0.4% calcium phytate and 0.4—1.5% calcium chloride.

1.5. Determination of enzyme activity

The milk clotting and proteolytic enzyme activities were determined in the aqueous extract of the bran and in the powdered crude preparation (VÁMOS *et al.*, 1969a), and related to unit weight of the product, or unit volume of the extract and hereafter this is called enzyme concentration.

Rennin concentration was expressed in Soxhlet Units per g or ml powdered preparation or extract (SU g^{-1} or SU ml^{-1}). 1 SU means the amount of enzyme necessary to coagulate 1 ml milk at 35°C in 40 min. The substrate used in activity determinations was 10% w/v rehydrated milk without addition of $CaCl_2$.

The substrate used for proteolytic activity measurement was casein and proteolytic enzyme concentration was expressed by the amount of casein (in mg) decomposed by 1 g preparation at pH 6.0 during 1 hour at 35°C.

From the concentration data the ratio of rennin to proteolytic activity was calculated (H). The ratio permits of drawing conclusions as to the proteolytic contamination of the preparation and in comparison to the corresponding data of calf rennet, to its suitability for use in cheese manufacture.

1.6. Experiments on trays

Aluminium trays of 36 · 57 · 5 cm size, fitted with cover, were used in the experiments. Each tray was layered with 600 g wheat bran, wetted and

sterilized. The bran to water ratio and the temperature and duration of sterilization were the same as in the flask-experiments. The bran nutrient layer on the trays was about 1.5 cm thick. Each tray was inoculated with 100 ml spore suspension and fermented for 5 days at 35°C. The trays were aerated through an opening in the center of the cover, provided with a joint to connect an aquarium pump. The latter aeration device transported about 1 litre air per minute through a sterile cotton-filter to the air space of the trays. Taking into account the size of the air space, this amounts to a ten-fold air exchange per hour.

At the end of the fermentation period 3 l water was used per tray to extract the enzyme. The aqueous extract was treated in the same way as in the flask-experiments. Each experiment was performed in 3 replicates.

2. Results

2.1. Enzyme concentration of the aqueous bran extract as affected by cultivation period and temperature

The rennin concentrations of the aqueous extracts of bran cultures fermented in flasks as a function of cultivation period and temperature are shown in Fig. 1.

At 25°C the highest rennin concentration, 280 SU ml^{-1}, was achieved in cultures of 5 or 6 days. The 7-day yield was not significantly lower.

At 30°C there was no significant difference in the enzyme concentrations on the 4th, 5th, and 6th day of fermentation. The highest value was measured on the 5th day: 376 SU ml^{-1}. Similar results were obtained at 37°C, where 330 SU ml^{-1} was the highest value measured on the 4th day. Both at 30 and 37°C the enzyme concentration significantly decreased by the 7th day of fermentation. At both temperatures the values measured on the 3rd and 7th day were similar. There was no significant difference in the maximum values as measured at 30 and 37°C.

At 45°C no significant difference was observed between the enzyme concentrations obtained on the 4th, 5th and 6th day, however a very highly or highly significant difference was found between these concentrations and those measured on the 3rd or 7th days.

As seen in the figure all the values measured at 45°C are substantially lower than those obtained at 25, 30 or 37°C. On the other hand the values as measured at 25°C were substantially lower than those obtained at 30 and 37°C, except those of 7 or 6 (at 30°C) and 7 days (at 37°C).

2.2. *Influence of cultivation period and temperature on the enzyme concentration of the preparation obtained by precipitation*

It was found in the flask fermentation experiments that the rennin concentration of the crude preparation varied with time and temperature similarly to that of the aqueous extract (Fig. 2).

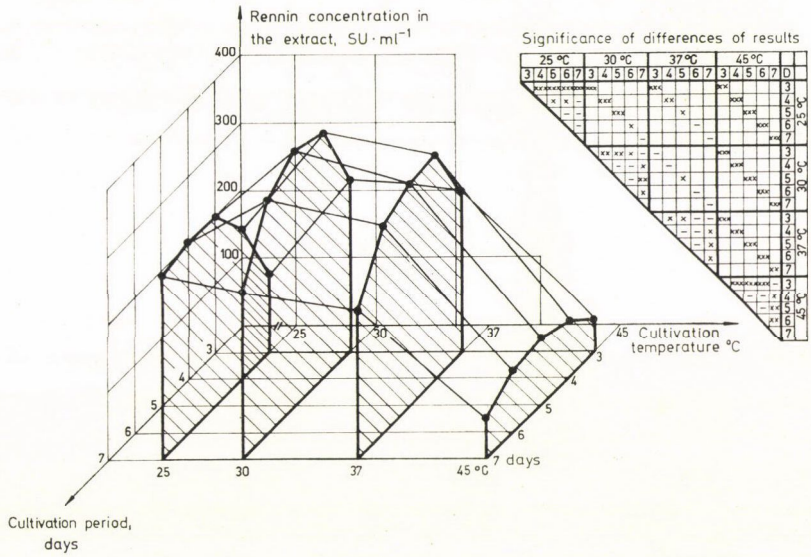

Fig. 1. Rennin concentration in the aqueous bran extract as a function of temperature and cultivation time (D= days). Conditions of fermentation: In flasks of 250 ml 20 g wheat bran were mixed with 14 ml tap water and sterilized at 133°C for 1 hour, inoculated to have a spore concentration of 3—5 · 10^{10} per 100 g bran. All experiments were carried out in 8 replicates. Significance of differences: × × × — significant at 99.9% probability level; × × — significant at 99.0% probability level; × — significant at 95.0% probability level; — — difference not significant

The highest rennin concentration was obtained on the 4th day at 30°C and this was: 23 000 SU g^{-1}. However the differences between this value and those obtained on the 3rd, 5th and 6th days of fermentation at 30°C were not significant. Similar values were obtained at 37°C on the 3rd and 4th day and at 25°C on the 3rd and 4th day. The values obtained for 3—6- or 4—6-day fermentations at 45°C were significantly lower than those obtained at 30 and 37°C or at 25°C, respectively.

The effect of cultivation time and temperature on the proteolytic enzyme concentration was similar to their effect on rennin concentration. The conditions found favourable for the biosynthesis of milk clotting enzyme (30—37°C, 4—6 days) resulted in a proteolytic enzyme concentration of 1600—2300 U g^{-1} and differences between the results were not significant.

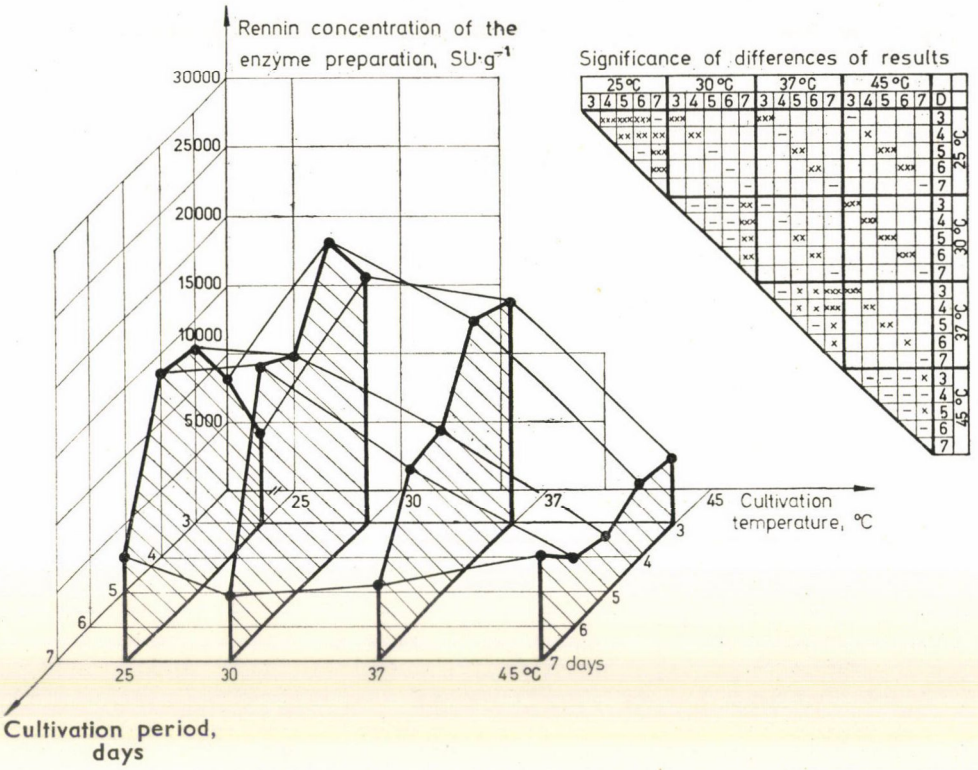

Fig. 2. Rennin concentration in the precipitated enzyme preparation as a function of temperature and cultivation period. Conditions of fermentation as in Fig. 1. Every sample was precipitated with 3 volumes of technical grade cooled methanol, washed in absolute ethanol, dried at room temperature and ground. Rennin concentration was determined by Soxhlet's method on 10% rehydrated milk substrate, without addition of $CaCl_2$. 1 Soxhlet Unit (SU) means the amount of enzyme necessary to coagulate 1 ml milk at 35° C in 40 min. For significance of differences see legend to Fig. 1

2.3. Investigation of the effects of additives

The compounds as specified in para. 1.4. did not increase the enzyme producing capacity of the mould strain, thus they were not utilized in further experiments.

2.4. Fermentation in trays

Parallel to the fermentation trials on trays at 35°C for 5 days, experimental fermentation was carried out in flasks. The average rennin concentration of the aqueous extract of the trays was 624 U ml^{-1} ($s = \pm 37$; $n = 3$) while in the case of flask cultures it was 597 U ml^{-1} ($s = \pm 24$; $n = 5$). According to Student's t test the two results do not differ significantly.

The enzyme concentrations and their ratio are compared to similar data pertinent to Meito Rennet and some calf rennet preparations in Fig. 3.

As seen in the figure the differences between proteolytic and rennin concentrations and the ratio of these concentrations, respectively, of the preparations obtained from flask cultures and of tray cultures, were not significant.

If the characteristics of the crude enzyme preparation, as shown in the figure, are compared to similar characteristics of Meito Rennet it becomes apparent that the rennin concentration of the former is about two thirds of the latter and the proteolytic concentration 1.7—2-fold; accordingly, the H value is just below one third. The same data compared to those of calf rennet used in cheese manufacture are 2.5- and 7-fold, respectively, and the H value 0.3-fold.

To compare these data with the values obtained by IWASAKI and co-workers (1967) would be difficult because the calcium content of the milk used as substrate differed and accordingly the milk clotting activity of the enzyme preparations was differently affected.

3. Conclusions

Under the given experimental conditions with the *Mucor pusillus* Lindt mould strain an enzyme preparation of milk clotting activity could be produced on a nutrient medium of very simple composition. The optimum conditions of fermentation were cultivation at 35°C for 5 days with a 10-fold aeration per hour. From a tray containing 600 g bran a crude powdered enzyme preparation sufficient to curdle 900 l milk was obtained in this way.

The rennin concentration of the preparation was 20 000—60 000 SU g^{-1}, thus in a number of cases it did not reach the value of 50 000 SU g^{-1}, required in the dairy industry from calf rennet. Another unfavourable characteristic was an about 5—7-fold proteolytic enzyme concentration as compared to calf rennet, which might cause a poorer cheese consistency, or even a bitter taste.

About 35% of the milk clotting activity of the aqueous enzyme extract was lost during further processing. 1 kg bran yields an amount of enzyme sufficient to clot 1 500 l milk. In case of an enzyme concentration of 50 000 SU g^{-1} this means a 3% yield related to the bran. Probably the loss occurring on precipitation of the enzyme may be reduced. This problem, however, was not studied, since the primary aim was to check the enzyme producing capacity of the strain.

On comparing the experiences presented with data found in the literature (IWASAKI *et al.*, 1967) the productivity of the strain studied seemed to be favourable.

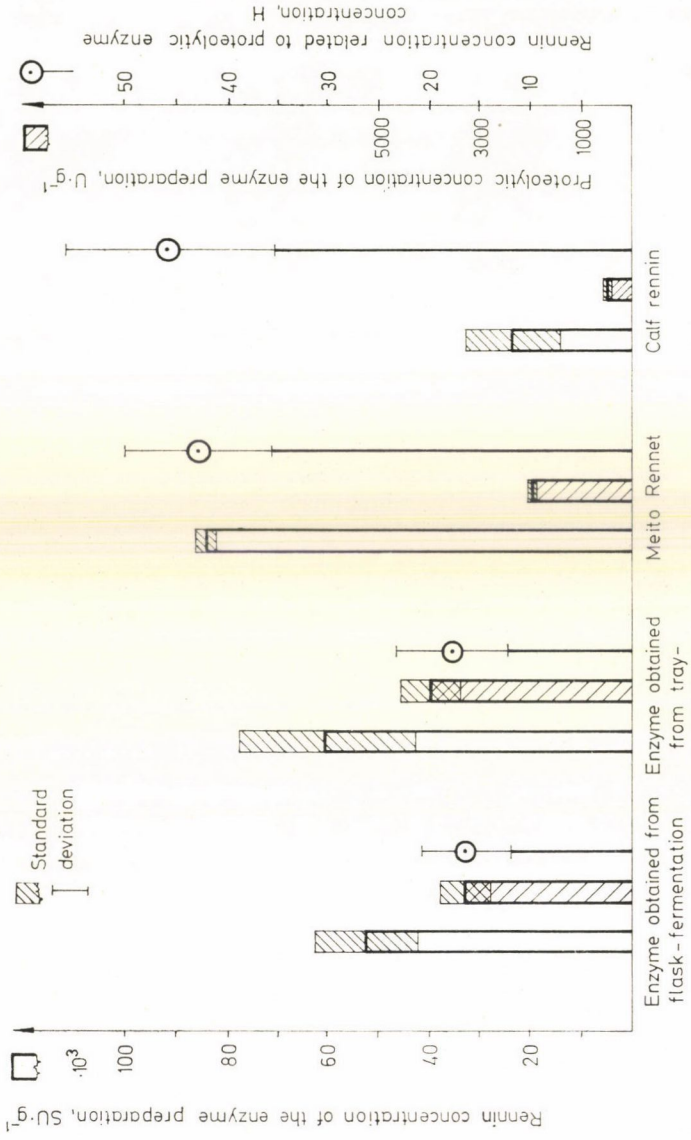

Fig. 3. Rennin and proteolytic enzyme concentration in the preparations and the ratio of the two enzymes. Rennin concentration was determined as described in legend to Fig. 2. Proteolytic activity was determined on casein substrate. Unit proteolytic enzyme concentration (U g^{-1}) is the amount of casein (in mg) decomposed by 1 g preparation

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INVESTIGATIONS INTO THE PRODUCTION OF MILK CLOTTING ENZYME PREPARATIONS

PART II. — BIOSYNTHESIS OF THE ENZYME BY *MUCOR PUSILLUS* LINDT IN SUBMERGED CULTURE

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The most favourable conditions of submerged fermentation of *Mucor pusillus* Lindt to produce milk clotting enzyme were as follows: nutrient medium 5% wheat bran, sterilized at 133°C for 1 hour, pH 5.7—6.0, inoculum 5—7%, shaken for 24 hours, aeration of the culture for 48 hours under shaking at a dissolution rate of 17 *mM* $O_2\,l^{-1}h^{-1}$. The maximum rennin enzyme concentration of the fermentation broth obtained by this method was 860 SU ml^{-1} and that of the powdered crude preparation, obtained by precipitation with three volumes of —20°C methanol, 72 700 SU g^{-1}.

Comparison of these results with those obtained in surface fermentation, as reported in a previous paper (POZSÁR-HAJNAL *et al.*, 1974), shows that the highest rennin concentration in the fermentation broth was obtained with vegetative inoculum in 48 hours instead of 5 days as found in surface fermentation. In surface fermentation 100 g bran yielded 3 g preparation of 50 000 SU g^{-1} rennin concentration, sufficient to coagulate 150 l milk, while in submerged fermentation the same amount of bran yielded 11 g preparation, sufficient to coagulate 550 l milk. In submerged fermentation the amount of enzyme synthetized per day is about tenfold as compared to that obtained by surface fermentation. Since, however, the relative proteolytic activity of the preparation produced by submerged fermentation is substantially higher, this preparation in its crude state is not suitable for cheese manufacture.

In a previous paper (POZSÁR-HAJNAL *et al.*, 1974) an account was given of investigations into the enzyme producing capacity of *Mucor pusillus* Lindt in surface culture.

Since these results were promising it seemed reasonable to investigate the possibility of producing the enzyme in submerged fermentation as well. The subject of the investigations described here was aimed at selecting an appropriate medium for submerged fermentation. Of the great number of media tested one containing wheat bran and another containing malt and milk were found most suitable to produce enzyme of milk clotting activity. In these experiments some of the media were inoculated with vegetative cells, others with spores. The effect of the fermentation period, the age of the inoculum and its quantity, the composition of the medium used to prepare the inoculum and the conditions of sterilization of the medium were studied.

1. Materials and methods

1.1. Medium based on malt

The malt extract of 7% w/v was sterilized in an autoclave for 5 minutes at 133°C, then separated from the precipitate by filtration while warm. The refractometer value of the medium was set at 5% and it was sterilized for 30 minutes at 133°C. 80 ml of this liquid medium was made up to 100 ml with 20 ml of 10% freshly rehydrated milk and heat treated for 45 minutes in steam stream. The pH of the medium was 6.0.

Fermentation was carried out in 500-ml Erlenmeyer flasks containing 100 ml medium each.

1.2. Wheat bran medium

100-ml portions of a wheat bran slurry of 5% w/v were sterilized in Erlenmeyer flasks of 500 ml for 1 hour at 133°C (SOMKUTI & BABEL, 1967, 1968). Generally the pH of the nutrient medium was set at 6.0—6.2, however, in certain cases, to avoid bacterial contamination *(Bacillus subtilis)*, it was set at 5.0.

1.3. Inoculum

The spores were washed off the agar slant cultures of various ages with a physiological salt solution. The concentration of the suspension thus obtained was $10^7—10^8$ ml^{-1}. 5 ml spore suspension was used to inoculate 100 ml nutrient medium.

The inoculum consisting of vegetative cells was prepared by shaking an inoculated 5% bran or bran extract medium for 24 hours. It was applied in concentrations of 5—25% v/v of the fermentation medium.

1.4. Fermentation

Fermentation was carried out at 30°C in aerated shake cultures. The rate of oxygen dissolution was in the case of the malt nutrient 12.5 *mM* $O_2\,l^{-1}h^{-1}$, in the case of wheat bran 17 *mM* $O_2\,l^{-1}h^{-1}$ (determined by the sulphite oxidation method).

The milk clotting activity of the fermentation broth was checked daily.

1.5. Separation of the enzyme from the fermentation broth

The enzyme was precipitated in the fermentation broth by a three-fold volume of methanol cooled to —20°C. In some cases, the enzyme was obtained from the bran medium by fractionated precipitation with —20°C methanol

using 750 ml methanol to 1000 ml fermentation broth to remove inactive proteins. The precipitate was centrifuged and 2300 ml methanol was added to the supernatant to precipitate the enzyme protein.

The enzyme precipitate was treated further on in the same way as in the case of surface fermentation (POZSÁR-HAJNAL *et al.*, 1974).

Besides rennin concentration the proteolytic enzyme concentration was also determined in the powdered enzyme preparations.

1.6. Determination of the enzyme concentration

The milk clotting activity of the fermentation broths and powdered preparations was determined by Soxhlet's method (IWASAKI *et al.*, 1967). Enzyme concentration is the amount of milk, in ml, coagulated by 1 g of enzyme preparation or 1 ml of fermentation broth at 35°C during 40 minutes. (Rennin concentration: SU g^{-1} or SU ml^{-1}, where SU = Soxhlet Unit.)

The proteolytic enzyme concentration was determined by the method developed earlier at this Institute, using casein as the substrate (VÁMOS *et al.*, 1969), and is expressed by the amount of casein (in mg) degraded by 1 g enzyme preparation at pH 6.0 during 1 hour at 35°C (U g^{-1}).

2. Results

2.1. Influence of fermentation time

The results obtained on malt nutrient to establish the effect of the fermentation period are summarized in Table 1.

Table 1

Results of submerged fermentations on medium based on malt

	Fermentation period							
	Rennin concentration							
	1 day		2 days		3 days		4 days	
	Fermentation liquor SU ml^{-1}	Crude preparation SU g^{-1}	Fermentation liquor SU ml^{-1}	Crude preparation SU g^{-1}	Fermentation liquor SU ml^{-1}	Crude preparation SU g^{-1}	Fermentation liquor SU ml^{-1}	Crude preparation SU g^{-1}
Parallel	400	14 545	266	10 000	888	25 000	728	23 529
fermen-	500	13 333	266	13 333	470	25 000	728	25 000
tations	320		333		308		133	
$\bar{x}$	406	13 939	288	11 666	555	25 000	529	24 264
s	90		35		290		343	

$\bar{x}$ = mean
s = standard deviation
SU = Soxhlet unit
U = proteolytic unit
pH = 6.0—6.4, 30°C

As seen in the table the highest rennin concentration was achieved by 3- and 4-day fermentations, both in the fermentation broth and in the preparation obtained from it. Therefore in further experiments 3-day fermentation was applied. The average rennin concentration of 7 parallel fermentation broths was 554 $\pm$ 228 SU ml^{-1}. The precipitated preparations contained

Table 2

Results of submerged fermentations on 5% wheat bran medium

Fermentation period, days	Fermentation liquor	Powdered preparation				H
		Rennin concentration SU g^{-1}		Proteolytic concentration U g^{-1}		
	Rennin concentration SU ml^{-1}	I	II	I	II	
		Fraction		Fraction		
3	123	4 353	62 500	67 567	92 592	0.68
	571					
4	222	3 538	9 200	14 285	16 668	0.55
	444		8 209*		14 285*	0.57
5	132	8 738	10 318	53 996	32 211	0.32
	222					
6	40					

* = precipitation was carried out with methanol (1 : 3) without fractionation
H = ratio of rennin to proteolytic enzyme concentration
SU = Soxhlet unit
U = proteolytic unit
pH = 6.0—6.3, , 30°C

47 560 $\pm$ 29 950 SU g^{-1} rennin and 35 923 $\pm$ 14 732 U g^{-1} proteolytic enzyme concentration. The ratio of rennin concentration to proteolytic enzyme concentration (H) was: 1.35 $\pm$ 0.13.

Mycelium growth was slower on the bran medium, therefore fermentations on this medium were extended to 3—6 days. The results of these experiments are given in Table 2.

As seen, though the rennin concentration was relatively high after a 3-day fermentation, the ratio of the two enzymes on this medium was below 1 and this is unfavourable from the point of view of cheese manufacture.

Further experiments were aimed at shifting the biosynthesis of milk clotting and proteolytic enzymes in a more favourable direction by varying the preparation method and quantity of the inoculum. Both media were used in these experiments.

2.2. Influence of the age of inoculum.

The results obtained by using 1-, 2- and 10-week inocula, respectively, in fermentations of 3, 4 and 5 days are given in Table 3.

The rennin concentration values as seen in the table represent the mean of two parallel fermentation experiments.

Comparison of the data by mathematical statistical analysis (Student's t-test) showed the difference in the rennin and proteolytic enzyme concentrations of fermentations, carried out on identical media with inocula of different ages, for 3, 4 and 5 days, to be non significant at a probability level of 95%. Thus the fermentations carried out on identical media with inocula of various ages and for different periods may be considered as one statistical population each. Thus the mean value on malt medium of the rennin concentrations is 554 ± 167 in the fermentation liquor, $42\,173 \pm 14\,400$ in the powdered preparation and the proteolytic enzyme concentration of the latter is $65\,520 \pm 26\,500$. The corresponding values on bran medium are as follows: 678 ± 323, $36\,414 \pm 11\,480$ and $54\,556 \pm 17\,150$, respectively. The relative values of activities (H) are 0.78 ± 0.61, and 0.60 ± 0.34, resp.

Comparison of the results of experiments carried out on the two different media showed no significant difference in the rennin concentrations of the two fermentation liquors, or in the rennin and proteolytic concentrations of the preparations obtained from them, nor in the ratios of their activities.

Thus for further experiments wheat bran medium was used, which is easier to prepare. The inoculum was the spore suspension washed off cultures not older than 4—5 weeks.

2.3. Comparison of inocula containing vegetative cells or spores

The inoculum containing vegetative cells was prepared by 24-hour shake cultures on 5% wheat bran medium. Each flask containing 100 ml nutrient medium was inoculated with 5 ml spore suspension of $4 \cdot 10^8$ ml^{-1} concentration. The 5% wheat bran fermentation medium was set at pH 5.0, and 25 ml of inoculum, containing vegetative cells, was added to 100 ml medium.

For the fermentation experiments with spore-containing inoculum the same spore suspension was used as above, adding 5 ml of it to every 100 ml of nutrient medium. Results are given in Table 4.

It appears from the data that the relative proteolytic activity was not reduced by the use of a vegetative inoculum.

In order to simplify the preparation of nutrient medium the use of a 5% bran extract, set at pH 5.0, was also tested for fermentation. On this medium, however, no enzyme was formed, whether it was inoculated with spore suspension or vegetative cell suspension, only the growth of mycelia was observed.

Table 3

Biosynthesis of milk clotting and proteolytic enzymes as affected by the age of the spore inoculum

Medium	Age of inoculum, weeks	Fermentation period, days	Fermentation liquor	Powdered preparation		Ratio of concentrations H
			Rennin concentration* SU ml^{-1}	Rennin concentration* SU g^{-1}	Proteolytic concentration** U g^{-1}	
Milk + malt medium	1	3	888 640	40 000 42 105	75 000	0.55
		4	470 444	40 000 26 629	100 000	0.33
		5	444 500	27 586 30 769	75 000	0.41
	2	3	500 500	23 529 20 000	30 000	0.73
		4	888 888	66 666 50 000	80 000	0.73
		5	571 615	44 444 42 105	75 000	0.58
	10	3	500 533	33 333	75 000	0.44
		4	400 364	61 528 66 666	27 700	2.31
		5	444 400	44 444 57 142	52 000	0.98
Wheat bran medium	1	3	1 333 1 230	30 000 57 000	75 000	0.58
		4	727 666	72 727 50 000	75 000	0.86
		5	666 727	25 800 25 000	50 000	0.51
	2	3	666 615	86 863 19 047	30 000	0.93
		4	1 142 1 000	33 333 20 769	75 000	0.43
		5	666 666	44 444 40 000	38 000	1.11
	10	3	470 444	30 769	43 000	0.72
		4	320 320	32 000 33 333	47 000	0.70
		5	320 296	34 782 26 666	58 000	0.53

* = determined from two parallel experiments
** = determined from the mixture of two parallel preparations
pH = 6.0—6.6, 30°C; SU = Soxhlet unit; U = proteolytic unit

Table 4

Comparison of spore inoculum and vegetative cell inoculum on 5% wheat bran medium

Fermentation, days	Inoculum	Fermentation liquor	Powdered preparation		Ratio of concentrations H
		Rennin concentration SU ml^{-1}	Rennin concentration SU g^{-1}	Proteolytic concentration U g^{-1}	
3	Spores	666	40 000	44 600	0.90
	Vegetative cells	666	57 000	50 000	1.14
4	Spores	728	30 500	44 000	0.69
	Vegetative cells	666	30 500	42 300	0.72
5	Spores	400	25 000	30 000	0.83
	Vegetative cells	400	29 000	30 000	0.97

pH = 5.7—6.0, 30°C
SU = Soxhlet unit
U = proteolytic unit

2.4. Enzyme concentration of the fermentation liquor and of the powdered preparation as affected by cultivation time and quantity of vegetative inoculum

In order to reduce the inoculum used experiments were carried out on 5% wheat bran, set at pH 6.0, inoculated with vegetative cells incubated on a 5% bran extract for 24 hours. Results are shown in Table 5.

As seen in the table substantial change in the rennin concentration of the fermentation liquor was not achieved by inoculating various amounts of vegetative cells. The effect of the fermentation period was much more important. The highest rennin concentration was obtained by 2-day fermentation in the fermentation liquor inoculated with vegetative cells: 858 SU ml^{-1}. The difference between this value and the enzyme concentration of fermentation liquors after 3- or 4-day fermentation was significant ($t = 3.84$, DF = 4). The enzyme preparation obtained from 2-day fermentation liquors had a higher rennin concentration than those made of fermentation liquors of longer fermentation periods. The pH of the fermentation liquor increases with time and the enzyme concentration of the preparation obtained from fermentation liquors of identical enzyme concentration (in 3- and 4-day cultures) is steadily decreasing. However, as shown by the relative value of H, the proteolytic enzyme concentration of the preparations decreases practically parallel with the rennin concentration.

Table 5

Enzyme concentration of the fermentation liquor and of the powdered preparation as affected by the fermentation period and the quantity of vegetative inoculum

Fermentation period, days	Inoculum %	Fermentation liquor			Powdered preparation*		Ratio of concentrations H
		pH	Rennin concentration		Rennin concentration SU g^{-1}	Proteolytic concentration U g^{-1}	
			SU ml^{-1}	daily mean and standard deviation			
2	5	5.7	888	858.6 ± 46.4	72 772	75 000	0.97
	7	5.7	888				
	10	5.7	800				
3	5	5.7	727	731.0 ± 67.1	53 333	52 000	1.02
	7	5.7	800				
	10	5.7	666				
4	5	6.0	727	706.7 ± 35.1	34 782	38 000	0.92
	7	6.0	727				
	10	6.0	666				
5	5	6.6	333	320.3 ± 12.5			
	7	6.6	308				
	10	6.6	320				

* = the powdered preparation was obtained from the combination of 3 parallel fermentation liquors
SU = Soxhlet unit
U = proteolytic unit

3. Conclusions

To produce a milk clotting enzyme preparation in submerged culture — using the described media and culturing conditions — the following seemed suitable: (a) 5% w/v wheat bran medium, inoculated with a 24-hour 5% vegetative cell suspension and (b) a fermentation time of 2 days. In this way using 100-ml shake cultures, in flasks of 500 ml, enzyme preparations of 70 000 SU g^{-1} rennin concentration were obtained. When the experiment was repeated on a larger scale (in 1 000-ml flasks 350 ml medium each and 7% inoculum) 15 l fermentation liquor yielded 85 g powdered enzyme preparation of 50 000 SU g^{-1} rennin concentration and 58 000 U g^{-1} proteolytic enzyme concentration, and the ratio of the activities was 0.86. 42% of the rennin concentration of the fermentation liquor was recovered in the powdered preparation.

On the whole the rennin concentration of the preparation obtained by using a vegetative cell suspension as inoculum and a fermentation period of 48 hours, was satisfactory. However, because of its high proteolytic enzyme

concentration the preparation is not suitable for cheese manufacture. Since the modification of the fermentation conditions failed to bring about a more favourable composition of the preparation, *i.e.* to reduce its relative proteolytic concentration, it may be assumed that in submerged culture the microorganism used synthetizes an enzyme differing from that synthetized in surface culture.

Table 6 contains the results obtained both in surface (POZSÁR-HAJNAL *et al.*, 1974) and submerged cultures.

Table 6

Comparison of the surface and submerged fermentation of Mucor pustillus Lindt

Characteristics of the fermentation / Method of fermentation	Fermentation period, days	Maximum rennin concentration		Proteolytic concentration of the powdered preparation U g^{-1}	Ratio of concentrations H	The amount of powdered preparation of 50 000 SU g^{-1} rennin concentration obtained from 100 g bran	
		In the fermentation liquor SU ml^{-1}	In the powdered preparation SU g^{-1}			g	g day^{-1}
Surface	5	400	58 000	3 860	15	3	0.6
Submerged	2	860	72 700	75 000	0.97	11	5.5

SU = Soxhlet unit
U = proteolytic unit

It appears that the enzyme preparation obtained by submerged fermentation is of relatively high rennin concentration and of higher yield than that obtained in surface culture.

Thus it seemed expedient to try to reduce the proteolytic enzyme concentration of the preparation by selective precipitation and sorption methods. Relevant investigations were reported by MORVAI elsewhere (1971).

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CONTRIBUTION TO THE THREE-DIMENSIONAL PRESSING THEORY AND ITS ONE-DIMENSIONAL APPLICATION

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The fundamentals and mathematical analysis of three-dimensional pressing theory are presented. Previous concepts of *Terzaghi*, *Heinrich*, *Biot*, *Carman*, *Tiller*, *Körmendy* are summarized and improved further. Vector and tensor calculations are used to have simple and concise treatment. The mathematical formulation results in a system of differential equations together with the initial and boundary conditions.

It is also stated that, in case the position-time relation of each solid element is known as the solution of a pressing problem, the calculation of the porosity as a function of position and time can be performed.

Hereafter a one-dimensional arrangement is discussed that results in a comparatively simple non-linear partial differential equation of second order and parabolic type, being analogous to the differential equation of diffusion. Based on the differential equation the pressing coefficient is introduced. The equivalent of the pressing coefficient is diffusivity. Comparing the pressing coefficient to *Terzaghi*'s consolidation coefficient it is demonstrated that the latter implies certain negligations.

Comparison is also made to *Shirato*'s modified consolidation coefficient and demonstrated, that it differs only in a constant factor from the pressing coefficient.

Some practical consequences of the theory are mentioned. The well-pressable material must have both well-deformable solid phase and low resistance of flow between the solid and liquid phases. Thus optimum grade of comminution exists for fruits. The time to reach a definite percentage yield of the liquid phase is proportional to the square of the initial thickness of the pressed cake. Therefore apparatus with slight initial cake thickness are advantageous.

Pressing is an operation to separate materials composed of solid and liquid (fluid) constituents (phases). Both phases are supposed to be contiguous, *i.e.* each element or particle of a phase is in contact with the others of the same phase, each element can transfer stresses to the neighbouring ones. Separation is caused by forces applied to the material, namely body forces and forces acting on surfaces with the meaning used in continuum mechanics. During the pressing operation the volume of the material is reduced and the liquid phase flows out through a definite part of the surface that confines the material. This part of the surface is impermeable for solids, while other parts of the surface are impermeable for both phases.

A simple example of the pressing operation is the squeezing out of water from an elastic sponge.

Pressing is closely related to the filtration and sedimentation operations. In all three processes the two phases are in relative motion caused by forces.

However, in the case of the two latter operations the material at the beginning consists of solid particles that are segregated each from the others inside the fluid constituent. As filtration or sedimentation proceeds in time a filter cake or sediment layer is building up and roughly the same considerations shall be applicable to the cake or layer as to the pressing operation in the one-dimensional case or in the case of cylindrical symmetry, etc.

The pressing operation is related both to fluid mechanics and stress analysis. The forces applied to the material cause stresses and elastic or plastic deformation, while the fluid phase will be forced to flow in the pores towards the exit surface.

Works published on pressing can be divided into the following main groups:

a) Contributions directly on the pressing operation such as the works of GURNHAM and MASSON (1946), GURNHAM (1950), KÖRMENDY (1963, 1964, 1965, 1968, 1972), SCHALLER (1965), SCHALLER and KNORR (1972), SHIRATO and MURASE (1970).

b) Filtration and sedimentation theory with relations to pressing such as the works of CARMAN (1938), GRACE (1953), SHIRATO *et al.* (1965, 1970a, 1970b), STJENITZER (1955), TILLER (1958).

c) Consolidation theory in soil mechanics such as the works of BIOT (1941), HEINRICH (1938), TAYLOR (1960), TERZAGHI (1925, 1954).

Authors in the chemical engineering field prefer the term "expression" instead of "pressing", and when the pressing operation is related to the filtration or sedimentation operations they use the term "consolidation". This term was generated in the field of soil mechanics and TERZAGHI (1925) can be regarded as the founder of the consolidation theory in soil mechanics. KÖRMENDY (1963, 1964, 1965) elaborated the one-dimensional pressing theory quite independently and in a more generalized form; however, the basic concepts of the two theories coincide.

A generalized pressing theory is considered to have a number of very different applications. Apart from process engineering (chemical and food industries) and soil mechanics, there are different shrinkage processes too. *E.g.* the shrinkage of meat during cooking, connected with water loss, or the shrinkage of liquid containing porous solids due to heat contraction.

Some consequences of theoretical results will be discussed in the last part of this communication.

1. The basic conditions and principles of the pressing theory

In the next paragraphs the author will recount the basic conditions and principles of the pressing theory. One part of these principles can be regarded as axioms.

1.1. Principles which enable the use of methods applied in continuum mechanics

It will be supposed, that both the solid and liquid constituents in the pressed material are contiguous and each can be treated as a continuum. Let us consider a cubic element in the material, its sides being parallel to the coordinate planes. The side lengths of the element will be denoted with dx, dy, dz. Let us suppose that these side lengths are great enough compared with the microscopic discontinuities to treat each constituent as a continuum. On the other hand the side lengths are small enough compared with the whole geometric arrangement to use them as infinitesimals. The above assumptions are in accordance with BIOT (1941).

1.2. Continuity equations

Evidently the continuity equations can be applied to both phases as a consequence of para. 1.1. The continuity equations will be presented here in forms which contain the porosity (ε) instead of the apparent densities of the phases. Thus they will take the form:

$$\operatorname{div}(1-\varepsilon)\,\bar{u} - \frac{\partial \varepsilon}{\partial t} = 0 \tag{1}$$

and

$$\operatorname{div} \varepsilon \bar{v} + \frac{\partial \varepsilon}{\partial t} = 0. \tag{2}$$

In the above equations $\bar{u}$ is the true velocity vector of an infinitesimal particle in the solid phase, $\bar{v}$ is the same in the liquid phase and t is the time. (See List of symbols, p. 108.)

Adding Equ. 1 to Equ. 2, the following relation results:

$$\operatorname{div} \varepsilon (\bar{v} - \bar{u}) + \operatorname{div} \bar{u} = 0. \tag{3}$$

It was assumed that both phases are incompressible, though deformable. Thus ϱ_f, the true density of the liquid phase and ϱ_s, the true density of the solid one are supposed to be constant.

In Equ. 3 the vector $\varepsilon(\bar{v} - \bar{u})$ means the apparent relative velocity vector of liquid to solids, its direction and sense evidently equals the relative velocity $\bar{v} - \bar{u}$. The size (or absolute value) of the apparent relative velocity vector is equal to the volume rate of the fluid phase flowing through a unit surface element perpendicular to the vector's direction and moving with the velocity $\bar{u}$. Applying the symbol $\bar{v}_F$ to the apparent relative velocity vector of liquid to solids we can write:

$$\bar{v}_F = \varepsilon (\bar{v} - \bar{u}) \tag{4}$$

and thus Equ. 3 obtains the following form:

$$\operatorname{div} \bar{v}_F + \operatorname{div} \bar{u} = 0. \tag{5}$$

1.3. Physical properties of the liquid phase and its stress conditions

The separated fluid constituent will be assumed to be incompressible and Newtonian. The whole pressing operation will be regarded as isothermal and therefore the fluid phase regarded having a constant dynamic viscosity (μ) and true density (ϱ_f). On the other hand the fluid phase when filling the pores of the material will be treated as a special fluid, whose density depends on the porosity and is unable to transfer tangential (shear) stresses like ideal fluids. Thus its stress condition can be characterized by an apparent (hydraulic) pressure (p_f). Its apparent stress tensor can be given by the following equation: $\bar{\bar{\Phi}}_f = -p_f \bar{\bar{I}}$ where $\bar{\bar{I}}$ is the unit tensor.

So far there have been two different concepts on the relation between the apparent and the true (hydraulic) pressures of the liquid constituent.

a) According to TILLER (1958) and others the apparent pressure is considered equal to the true one, thus

$$p_f = p. \tag{6}$$

This supposition results from such a microscopic arrangement, where the solid particles are sphere-like and forces are transferred by a number of contact points between them. Moreover, when one refers to stresses inside the material these are always thought to act on such planes that contain only the contact points without intersecting the solid particles.

b) The other assumption, presumably originated by CARMAN (1938), was applied among others by STJENITZER (1955), TERZAGHI (1954), KÖRMENDY (1963, 1964) too.

According to this:

$$p_f = \varepsilon p. \tag{7}$$

This means that the apparent pressure is lower than the true (hydraulic) pressure and the proportionality factor is just the porosity. According to this concept the reference plane of stresses inside the material intersects both phases, and the liquid area related to the total intersected area is just equal to the porosity.

It is easy to understand that the latter assumption is more general and in conformity with the principles described in para. 1.1.

1.4. Strain-stress relations of the solid phase

It will be assumed that the strain-stress relations of the solid phase, in case the liquid phase is free from stresses (*i.e.* p and p_f are equal to zero), are known. This means that the functional relationship between the stress tensor of the solid phase ($\overline{\overline{\Phi}}_c$), the tensor of deformation ($\overline{\overline{S}}_c$) and the tensor of the rate of deformation ($\overline{\overline{V}}_c$) is known.

It would be misleading to use the above functional relationship in the case of complete separation of the two phases, because the lack of the presence of the liquid phase might change the mechanical (stress) characteristics of the solid one.

Stresses due to $\overline{\overline{\Phi}}_c$ will be named deformation stresses of the solid phase.

1.5. Stresses transferred by the solid phase and the resulting stress

In accordance with para. 1.3 tangential stresses in the material can be transferred only by the solid phase. Stresses transferred by the solid phase during the pressing operation will be due to a stress tensor $\overline{\overline{\Phi}}_s$, which generally differs from $\overline{\overline{\Phi}}_c$ in para. 1.4. A stress calculated with $\overline{\overline{\Phi}}_s$ is a force transferred by the solid phase and acting upon unit total cross sectional area.

The sum of the stresses transferred by both phases is called the resulting stress. Thus the resulting stress tensor $\overline{\overline{\Phi}}$ can be calculated of the following equation:

$$\overline{\overline{\Phi}} = \overline{\overline{\Phi}}_s + \overline{\overline{\Phi}}_f = \overline{\overline{\Phi}}_s - p_f\overline{\overline{I}}. \tag{8}$$

1.6. Relation between different stresses in the pressed material

Under the present paragraph we shall discuss the relation between the deformation stresses of the solid phase, the stresses transferred by the solid phase and the hydraulic pressure in the liquid phase.

It will be assumed that the deformation stress of the solid phase is equal to the difference of the resulting stress and the true hydraulic pressure in the liquid phase. The above assumption can be expressed by the following equation between the stress tensors:

$$\overline{\overline{\Phi}}_c = \overline{\overline{\Phi}} - (-p\overline{\overline{I}}) = \overline{\overline{\Phi}}_s - p_f\overline{\overline{I}} + p\overline{\overline{I}}. \tag{9}$$

Equ. 9 is a consequence and generalization of former theoretical and experimental results. Though in a more simple form, it was applied by CARMAN (1938) and STJENITZER (1955) and referred to as experimentally validated by TERZAGHI (1954). Equ. 9 obviously shows that the deformation stress of the solid phase will be equal to the stress transferred by the solid phase only if

the assumption under para. 1.3a is valid and thus Equ. 6 can be combined with Equ. 9.

On the other hand applying the hypothesis under para. 1.3b the following relation will be obtained between the two stress tensors, combining Equ. 7 and Equ. 9:

$$\bar{\bar{\Phi}}_c = \bar{\bar{\Phi}}_s + (1 - \varepsilon)\, p\bar{\bar{I}}. \tag{10}$$

To clarify the former concepts let us consider a cylindrical receiver which is filled with the solid-liquid mixture and is closed by a well-sealed piston on the top end.

Initially, applying no force on the piston, the material can be regarded as stress-free. Thus, considering only planes parallel to the piston surface of size A: $p = 0$, $p_f = 0$, $p_s = 0$, $p_c = 0$. Hereafter let us apply the force P_0 on the piston, it induces the resulting pressure $p_0 = \dfrac{P_0}{A}$. As both phases are incompressible no deformation will appear although stresses have considerably increased inside the material due to P_0.

Applying the considerations of para. 1.3a, the whole pressure exerted by the piston will be transferred by the liquid phase alone to the bottom of the cylindrical receiver, as $p_c = p_s = 0$.

Applying para. 1.3b the liquid phase will transfer only the part $p_f = \varepsilon p_0$ and the solid phase the part $p_s = (1 - \varepsilon)\, p_0$ to the bottom, however, $p_c = p_0 - p = 0$ again as p, the true pressure in the liquid is equal to p_0.

1.7. Basic equations of motion

The equation that states the equilibrium of different forces per unit volume, acting upon an infinitesimal element of the solid phase, moving with a velocity vector $\bar{u}$ and an acceleration $\dfrac{d\bar{u}}{dt}$, is the following:

$$\bar{\bar{\Phi}}_s \nabla + \varrho_s (1 - \varepsilon)\, \bar{a} + \bar{R} - \varrho_s (1 - \varepsilon) \frac{d\bar{u}}{dt} = 0. \tag{11}$$

Here ϱ_s is the true density of the solid phase, $\bar{a}$ is the body force acting upon unit mass, $\bar{R}$ is a force depending on the mutual effect of the two phases and acting on unit volume. ∇ is the vector operator "nabla" (note: tensors will be always written prior to the "nabla", as in Equ. 11, to emphasize the operator is a vector).

A similar equation is valid for the fluid phase:

$$-\overline{\mathrm{grad}}\, p_f + \varrho_f \varepsilon \bar{a} - \bar{R} - \varrho_f \varepsilon \frac{d\bar{v}}{dt} = 0. \tag{12}$$

Here ϱ_f is the true density of the liquid phase, $\bar{v}$ is the velocity vector of an infinitesimal element, $\frac{d\bar{v}}{dt}$ is the acceleration of the element.

If inertial forces $\left(\text{being proportional to } \frac{d\bar{u}}{dt} \text{ and } \frac{d\bar{v}}{dt}\right)$ can be neglected and we add Equ. 11 and Equ. 12, the following relation will result:

$$\bar{\bar{\Phi}}_s \nabla - \bar{\text{grad}}\, p_f = -[\varrho_s(1-\varepsilon) + \varrho_f \varepsilon]\,\bar{a}. \tag{13}$$

The combination of Equ. 10 and Equ. 13 gives the following equation:

$$\bar{\bar{\Phi}}_c \nabla - \bar{\text{grad}}\, p = -[\varrho_s(1-\varepsilon) + \varrho_f \varepsilon]\,\bar{a}. \tag{14}$$

The former equation is based on para. 1.3b, however, if hypotheses under para. 1.3a were taken into account, the result would be Equ. 14 again. In the latter case the value of $\bar{\bar{\Phi}}_s$ in Equ. 13 shall be simply replaced by $\bar{\bar{\Phi}}_c$ and p_f by p.

1.8. The introduction of Darcy's law

The generalization of *Darcy*'s law can be expressed in the following form, in case the flow is laminar and isotropic conditions exist:

$$-\bar{\text{grad}}\, p = \mu\alpha\bar{v}_F - \varrho_f\bar{a}. \tag{15}$$

Here α is the specific resistance of flow. This equation was thoroughly discussed by former authors. Equ. 15 makes it possible to calculate the value of $\bar{R}$ in Equ. 11 and Equ. 12. It is worth mentioning that the value of $\bar{R}$ depends on whether para. 1.3a or 1.3b has been taken into account. This circumstance would need further examination and seems to be the consequence of the difference in the microscopic picture of the material.

1.9. Differential equations formulating the pressing problem

The following transformations can be elaborated based on the former paragraphs, and neglecting the body force:

Combining Equ. 15 with Equ. 5 and Equ. 1 the following relation will be gained:

$$\text{div}\left(\frac{1}{\mu\alpha}\bar{\text{grad}}\, p\right) = \frac{1}{1-\varepsilon}\cdot\frac{d\varepsilon}{dt}. \tag{16}$$

This can be developed into the following form by using x, y and z coordinates:

$$\frac{\partial}{\partial x}\left(\frac{1}{\mu\alpha}\cdot\frac{\partial p}{\partial x}\right)+\frac{\partial}{\partial y}\left(\frac{1}{\mu\alpha}\cdot\frac{\partial p}{\partial y}\right)+\frac{\partial}{\partial z}\left(\frac{1}{\mu\alpha}\cdot\frac{\partial p}{\partial z}\right)=\frac{1}{1-\varepsilon}\cdot\frac{d\varepsilon}{dt}, \qquad (17)$$

here $\frac{d\varepsilon}{dt}$ is the rate of variation of the porosity of a solid particle moving with the velocity $\bar{u}$.

This circumstance induces to seek the solution of a pressing problem with the help of *Lagrange*'s method as discussed later in para. 1.11.

Likewise Equ. 14 also can be developed into the following scalar equations:

$$\begin{aligned}
&\frac{\partial\sigma_x}{\partial x}+\frac{\partial\tau_{xy}}{\partial y}+\frac{\partial\tau_{xz}}{\partial z}-\frac{\partial p}{\partial x}=0\\
&\frac{\partial\tau_{yx}}{\partial x}+\frac{\partial\sigma_y}{\partial y}+\frac{\partial\tau_{yz}}{\partial z}-\frac{\partial p}{\partial y}=0\\
&\frac{\partial\tau_{zx}}{\partial x}+\frac{\partial\tau_{zy}}{\partial y}+\frac{\partial\sigma_z}{\partial z}-\frac{\partial p}{\partial z}=0.
\end{aligned} \qquad (18)$$

1.10. Initial and boundary conditions

The surface of the pressed material can be divided into two main groups. The first one contains those parts of the surface which are impermeable to both phases (see Fig. 1 surface sections 2 and 6).

Here the relative velocity vector of the phases is either zero, or its component perpendicular to the surface is zero. Thus the vector can be directed

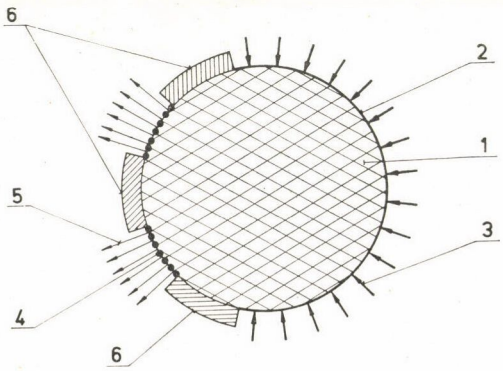

Fig. 1. A simplified general arrangement of the pressing operation. 1 — material to be pressed; 2 — surface under outer pressure; 3 — forces applied to the pressing operation; 4 — the surface of fluid outflow (filter cloth); 5 — direction of fluid outflow; 6 — rigid boundary surfaces

only in the tangential plane of the surface. This latter case has been disregarded in a previous work of KÖRMENDY (1968). Thus

$$\bar{n} \cdot (\bar{v} - \bar{u}) = 0. \tag{19}$$

Here $\bar{n}$ is a unit vector perpendicular to the surface and scalar multiplication is applied between the vectors.

The second group contains those sections of the surface, which retain the solid particles, but allow the fluid phase flow through (see Fig. 1 surface sections 4). These sections are mostly constructed in the form of a filter cloth or screen.

If the resistance of the filter cloth is negligible, then the true pressure in the liquid phase is the same on both sides of the filter cloth. If not, then the pressure drop through the filter cloth must be taken into account. Conditions are similar to the different boundary conditions used in the unsteady state cooling of solid bodies.

Forces and pressures acting on the outer side of both groups of surfaces must be known.

The initial condition gives the porosity as the function of position coordinates at the time of the beginning of the operation. Any other value which fully substitutes the porosity may be also used, such as void ratio (e) or specific volume of the solid phase (w).

1.11. Considerations on the solution of a pressing problem

Equ. 17 and Equ. 18 together with the stress-strain relation of the solid phase as discussed under para. 1.4 and the initial and boundary conditions under para. 1.10 give the possibility to solve a problem. This solution is reached if the position of every solid particle can be calculated as a function of time. However, the problem is also solved, if we know the porosity as the function of the position and time coordinates. The latter type of solution can be calculated with the help of the former one.

For example let us know the solution in the form

$$\bar{r} = \bar{r}(\bar{r}_i, t). \tag{20}$$

Here $\bar{r}_i$ is the position vector of a solid element at time $t = 0$, and called the "name" of the solid element. $\bar{r}$ is the position vector of the same element at time $t > 0$. The above relation makes possible to calculate the position of each particle with the "name" $\bar{r}_i$ at different values of time. Equ. 20 represents *Lagrange*'s method to describe position-time relations of the elements of the solid phase. The velocity of a solid element ($\bar{u}$) will be obtained by differen-

tiating $\bar{r}$ by the time t. The next step is to calculate $div\ \bar{u}$ and then the porosity through Equ. 1 either as the function of $\bar{r}_i$ and t or $\bar{r}$ and t.

The above considerations suggest, that it seems to be more useful to treat the problem according to *Lagrange*'s method and seek the solution by eliminating the components x, y and z of $\bar{r}$ by those of x_i, y_i and z_i, the components of $\bar{r}_i$ vector.

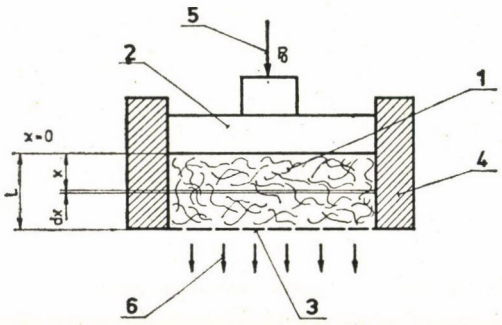

Fig. 2. An arrangement to press a layer of constant thickness. 1 — material to be pressed; 2 — pressure plate; 3 — filter cloth (screen); 4 — side wall; 5 — force applied to the pressing operation; 6 — fluid outflow

2. The pressing of a layer of constant thickness

In the following part the case will be discussed where the material is pressed by an arrangement according to Fig. 2. This case is important from the practical point of view and the most simple, because the use of only one position coordinate is enough for the mathematical treatment.

The solution is sought on the assumption that the same physical conditions are present at the side walls as in the inner parts of the material and gravitational and inertial forces are negligible.

This problem leads to the partial differential equation of second order:

$$\frac{\partial}{\partial x_i}\left(-\frac{1-\varepsilon}{\mu\alpha}\cdot\frac{dp_c}{d\varepsilon}\cdot\frac{\partial\varepsilon}{\partial x_i}\right)=\left(\frac{1-\varepsilon_i}{1-\varepsilon}\right)^2\cdot\frac{\partial\varepsilon}{\partial t} \tag{21}$$

(this equation was deduced from Equ. 17 through steps following in Equ. 23, Equ. 24 and Equ. 25), where x_i is the distance of a layer from the pressure plate at the beginning of the pressing operation. Thus the pressure plate was looked upon as fixed and the filter cloth moving towards it.

In Equ. 21 $p_c = -\sigma_x$ is the so-called "cake pressure". This pressure is due to the deformation of the solid phase. ε_i is the porosity at the beginning of the operation.

Initial and boundary conditions are the following:

$$\text{if} \qquad t = 0 \quad \text{and} \quad x_i \neq L_i, \quad \text{then} \quad \varepsilon = \varepsilon_i$$

$$\text{at} \qquad x_i = L_i \qquad \varepsilon = \varepsilon_0 = \varepsilon(p_0) \tag{22}$$

$$\text{at} \qquad x_i = 0 \qquad \frac{\partial \varepsilon}{\partial x_i} = 0.$$

Here ε_0 is the porosity after a very long time of pressing, its value depends on p_0. p_0 is the total pressure, that is the force P_0 divided by the area of the pressure plate. L_i is the thickness of the material at the beginning of the process (see Fig. 2). ε_i is the initial value of ε. Equ. 21 was deduced from Equ. 17. As here only one position coordinate is needed, Equ. 17 becomes

$$\frac{\partial}{\partial x}\left(\frac{1}{\mu\alpha} \cdot \frac{\partial p}{\partial x}\right) = \frac{1}{1-\varepsilon} \cdot \frac{d\varepsilon}{dt}. \tag{23}$$

The next step is to eliminate the position coordinate x on principles under para. 1.11. It is simply made with help of the following relation stating that the volume of the solid phase within the distance dx is the same as it was within the distance dx_i at time $t = 0$:

$$dx(1-\varepsilon) = dx_i(1-\varepsilon_i). \tag{24}$$

Thus we receive of Equ. 23:

$$\frac{\partial}{\partial x_i}\left(\frac{1-\varepsilon}{\mu\alpha} \cdot \frac{\partial p}{\partial x_i}\right) = \left(\frac{1-\varepsilon_i}{1-\varepsilon}\right)^2 \cdot \frac{\partial \varepsilon}{\partial t}. \tag{25}$$

The next step is to analyse the stress-strain relations inside the material. The study of the deformation of a prism whose base and cover planes are parallel to the pressure plate leads to the statement that $\sigma_x = -p_c$ is a principal stress and its value is constant along a plane parallel to the pressure plate at a given time. The principal stresses perpendicular to σ_x are equal and therefore no tangential stresses are present in the planes which are perpendicular to the pressure plate. These latter principal stresses have just the value to prevent the lateral deformation of the prism.

As a consequence of Equ. 9 the following relationship is valid along the cake:

$$p_c + p = p_0. \tag{26}$$

We suppose here that stresses due to the tensor $\overline{\Phi}_c$ depend only on the deformation, but are fully independent from the rate of deformation. So in

our case p_c determines the value of ε or vice-versa. If the deformation is not reversible, it shall be also conditioned that $\frac{\partial p_c}{\partial t} \geq 0$. The functional relation between p_c and ε can be stated in the following way: Pressing experiments are to be carried out in an apparatus built on the principle shown in Fig. 2. Pressing has to be continued with p_0 total pressure till no more fluid flows out of the cake ($p = 0$). In this case p_c will be equal to p_0 (see Equ. 26). The porosity of the cake must be measured in the latter condition.

Pressures are always measured above atmospheric level in the above discussions and the outer side of the filter cloth is thought to be under atmospheric conditions.

The above considerations permit the following substitution in Equ. 25:

$$\frac{\partial p}{\partial x_i} = \frac{\partial (p_0 - p_c)}{\partial x_i} = -\frac{\partial p_c}{\partial x_i} = -\frac{dp_c}{d\varepsilon} \cdot \frac{\partial \varepsilon}{\partial x_i}. \tag{27}$$

Thus we obtain the differential-equation of Equ. 21. The initial condition (Equ. 22) states that at the beginning of the operation all the material has the uniform porosity ε_i. The boundary condition at the filter cloth states, that the flow resistance of the cloth is negligible, thus p is equal to zero at the inner side of it and so ε takes just the value which belongs to $p_c = p_0$ here during the whole process. The boundary condition at the pressure plate was derived from Equ. 19.

Conditions at the wall of the cylinder are more complicated. Here the drag due to the friction of the material on the wall was negligated, though some authors, *e.g.* SHIRATO *et al.* (1968) took it into account. Other conditions were assumed to be the same as in other parts of the material.

We can introduce into Equ. 21 and Equ. 22 instead of ε either w, that is the volume of the solid-liquid mixture containing unit weight of solids, or e, which is the volume ratio of the liquid phase to the solid phase, *i.e.* the void ratio. These values depend on the porosity in the following way:

$$w = \frac{1}{\gamma_s (1 - \varepsilon)}, \tag{28}$$

$$e = \frac{\varepsilon}{1 - \varepsilon}. \tag{29}$$

Here $\gamma_s = \varrho_s g$ is the true specific weight of the solid phase, g is the gravitational constant. The author applies the value of w in the text which follows in order to derive Equ. 31 and Equ. 34 these being analogous to the differential equations of diffusion or heat conduction. Some authors, mainly in soil mechan-

ics, prefer the use of the dimensionless e. We can introduce instead of x_i the following dimensionless distance:

$$s = \frac{x_i}{L_i}. \tag{30}$$

The above ratio is at the same time equal to the weight ratio of the solid phase within the distance x_i relative to all the solid phase between the plate and the cloth.

If we transform Equ. 21 and Equ. 22 through Equ. 28 and Equ. 30, then the following expressions will be received:

$$\frac{\partial}{\partial s}\left[C\frac{\partial w}{\partial s}\right] = L_i^2 \frac{\partial w}{\partial t} \tag{31}$$

and

$$\begin{aligned} &\text{if} \quad t = 0 \quad \text{then} \quad w = w_i, \quad s \neq 1 \\ &\text{at} \quad s = 1 \quad w = w_0 = w(p_0) \\ &\text{at} \quad s = 0 \quad \frac{\partial w}{\partial s} = 0. \end{aligned} \tag{32}$$

Here

$$C = -\frac{w_i^2}{\mu \alpha w} \cdot \frac{dp_c}{dw}. \tag{33}$$

C will be called hereafter the *pressing coefficient* and its value is depending on w. This coefficient comprises two different physical characteristics of the pressed material. The value of $\mu \alpha w$ refers to the flow resistance between the phases, while $-\frac{dp_c}{dw}$ refers to the deformability of the solid phase. If $C =$ constant, then we receive

$$C\frac{\partial^2 w}{\partial s^2} = L_i^2 \frac{\partial w}{\partial t}. \tag{34}$$

The form of this equation equals the differential equation of heat conduction in solids. So the problem of pressing is formally the same as the cooling of a plate insulated on one side and of constant thickness.

The solution of our case will be sought in the form $\varepsilon = \varepsilon(x_i, t)$ or $w = w(s, t)$, functions with two independent variables. These solutions enable us to calculate the corresponding p_c or p values too, moreover Equ. 21 or Equ. 31 can be transformed to have p_c or p as the dependent variable instead of ε or w. Fig. 3 shows the variation of the above dependent variables at the beginning ($t = 0$), at process time t and after a very long process time ($t \to \infty$).

The difference between the application of the x coordinate and the x_i or s coordinates can be well seen.

At the beginning of the pressing operation w possesses the uniform and maximal value w_i throughout the cake, as it contains the maximal amount of liquid. As the operation proceeds the cake becomes thinner and thus the values of w are also decreasing. The material is more compressed at the filter

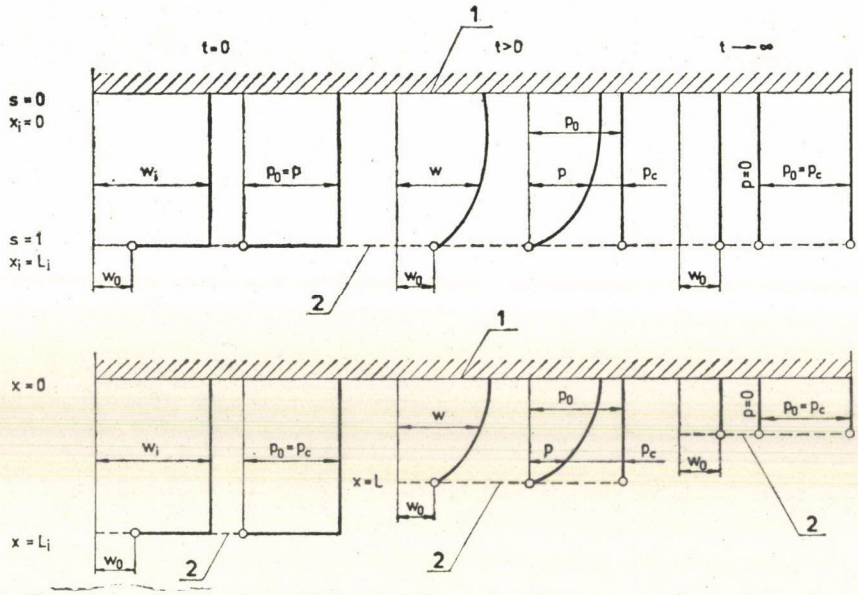

Fig. 3. Functions characteristic of the pressing operation. 1 — pressure-plate; 2 — filter cloth

cloth than at the pressure plate, because fluid flows out first of the layers beside the cloth. After a very long process time no more fluid leaves the cake and thus the value of w becomes uniform again and reaches the minimum value of w_0.

The pressing coefficient is almost equivalent to the consolidation coefficient proposed first by TERZAGHI (1954), but differs from it in some aspect. The original consolidation coefficient was created under certain negligations that have been avoided by the pressing coefficient. SHIRATO *et al.* (1970b) have developed some other modified forms of the consolidation coefficient, which differ from the pressing coefficient only in constant factors.

In Table 1 different forms of the pressing coefficient are shown parallel with those of the consolidation coefficient and modified consolidation coefficient. If the compression of the material is very slight and thus ε or w can be regarded as constant, then the substitution of ε, w or e by their initial value

Table 1

Different forms of the pressing and consolidation coefficients

Variant	Pressing coefficient	Consolidation coefficient	Mo fied consolidation coefficient of SHIRATO *et al.* (1970b)
ε	$C = -\frac{1-\varepsilon}{\mu\alpha}\left(\frac{1-\varepsilon}{1-\varepsilon_i}\right)^2 \frac{dp_c}{d\varepsilon}$	$C_v = -\frac{1-\varepsilon}{\mu\alpha}\frac{dp_c}{d\varepsilon}$ or $C_v = -\frac{1-\varepsilon_i}{\mu\alpha}\frac{dp_c}{d\varepsilon}$	$C_e = -\frac{\varrho_S^2(1-\varepsilon)^3}{\mu\alpha}\frac{dp_c}{d\varepsilon}$
w	$C = -\frac{w_l}{\mu\alpha}\left(\frac{w_l}{w}\right)\frac{dp_c}{dw}$	$C_v = -\frac{w_l}{\mu\alpha}\frac{dp_c}{dw}$ or $C_v = -\frac{w}{\mu\alpha}\frac{dp_c}{dw}$	$C_e = -\frac{1}{\varrho^2}\cdot\frac{1}{\mu\alpha w}\frac{dp_c}{dw}$
e	$C = -\frac{1+e_i}{\mu\alpha}\left(\frac{1+e_l}{1+e}\right)\frac{dp_c}{de}$	$C_v = -\frac{1+e_l}{\mu\alpha}\frac{dp_c}{de}$ or $C_v = -\frac{1+e}{\mu\alpha}\frac{dp_c}{de}$	$C_e = -\frac{\varrho_S^2}{\mu\alpha(1+e)}\frac{dp_c}{de}$
Dimension of the coefficient *e.g.*	$\frac{\mathrm{cm}^2}{\mathrm{sec}}$, $\frac{\mathrm{m}^2}{\mathrm{sec}}$, $\frac{\mathrm{m}^2}{\mathrm{hr}}$		$\frac{\mathrm{cm}^2}{\mathrm{sec}}\cdot\left(\frac{\mathrm{gram}}{\mathrm{cm}^3}\right)^2$

ε_i, w_i or e_i, or vice-versa, transforms the pressing coefficient into the consolidation coefficient. The pressing and the consolidation coefficients have the same dimension and these also coincide with the dimension of diffusivity and thermometric conductivity.

We have important evidence that C can not be constant in the case of comminuted fruits. Partly because the value of w alters on a large scale (the volume of the pressed material decreases sometimes to 1/5 of the original one), partly because experiments and industrial observations have so far indicated that higher total pressures in some cases lessen the rate of deformation. This latter observation indicates that in such cases the value of C decreases with increasing pressure.

As a conclusion, attention should be paid to some practical consequences:

a) Only that material can be regarded as well pressable, which consists of not only a well deformable solid structure, but also has a low flow resistance between the two phases. For example, an optimal grade of comminution exists for fruits, because the diminution of the particle size results in a more deformable structure but with a higher flow resistance.

b) The time to reach a definite percentage yield of the liquid phase is proportional to the square of the initial thickness of the pressed cake. This consequence was explained and validated experimentally in a previous work by KÖRMENDY (1965), together with other considerations on how the initial thickness of the cake affects the process. Thus designers should try to construct presses with a possibly slight initial cake thickness.

Previous works of KÖRMENDY (1963, 1964, 1965) treated the problem only as one-dimensional case and the results were essentially the same as here apart from some formal differences.

*

The author expresses his grateful thanks to Professor M. SHIRATO, University of Nagoya (Japan) for his kind help and prompt submittance of his recent works on filtration and expression.

List of symbols

Note: Dimensions in the brackets serve as exemplars.

$\bar{a}$ = body force acting on unit mass (cm/sec^2)
A = total cross-sectional area (cm^2)
C = pressing coefficient (cm^2/sec)
C_ε = modified consolidation coefficient (gram^2/cm^4 sec)
C_v = consolidation coefficient (cm^2/sec)
e = void ratio (volume of liquid per volume of solids in the pressed material)
e_i = the value of e at the beginning of the pressing process
g = gravitational constant (cm/sec^2)
$\bar{\bar{I}}$ = unit tensor
L = the thickness of the pressed material (cm)
L_i = the value of L at the beginning of the pressing process (cm)
$\bar{n}$ = unit vector, perpendicular to the surface

p = the true (hydraulic) pressure in the liquid phase (dyn/cm²)
p_c = cake pressure, *i.e.* the pressure due to the deformation of the solid phase in one-dimensional case (dyn/cm²)
p_f = the apparent (hydraulic) pressure in the liquid phase, or pressure transferred by the liquid phase (dyn/cm²)
p_0 = the total or resulting pressure in one-dimensional case (dyn/cm²)
p_s = pressure transferred by the solid phase (dyn/cm²)
P_0 = pressing force (dyn)
$\bar{r}$ = position vector (cm)
$\bar{r}_i$ = position vector of a solid element at time $t = 0$ (cm)
$\bar{R}$ = force generated by the mutual effect of the solid and liquid phases, acting on unit volume (dyn/cm³)
s = dimensionless coordinate as stated by Equ. 30, or the ratio of the weight of solids in a layer of the thickness x_i to the weight of solids in L_i, in one-dimensional case
$\bar{\bar{S}}_c$ = the tensor of the deformation of the solid phase
t = time (sec)
$\bar{u}$ = velocity vector of an infinitesimal element of the solid phase (cm/sec)
$\bar{v}$ = velocity vector of an infinitesimal element of the liquid phase (cm/sec)
$\bar{v}_F$ = apparent relative velocity vector of liquid to solids (cm/sec)
$\bar{\bar{V}}_c$ = the tensor of the rate of deformation of the solid phase (1/sec)
w = the volume of the pressed material containing unit weight of solids (cm³/pond)
w_i = the value of w at the beginning of the pressing process (cm³/pond)
w_0 = the value of w after a very long time of pressing due to $p_c = p_0$ (cm³/pond)
x, y, z = position coordinates, components of the position vector $\bar{r}$ (cm)
x_i, y_i, z_i = components of the position vector $\bar{r}_i$ (cm), or x_i is the distance from the pressure plate at the beginning of the pressing process
α = specific resistance (1/cm²)
γ_s = true specific weight of the solids (pond/cm³)
ε = porosity (volume of liquid per total volume of the pressed material)
ε_i = the value of ε at the beginning of the pressing process
ε_0 = the value of ε after a very long time of pressing due to $p_c = p_0$
$\bar{\bar{\Phi}}$ = the tensor of the resulting stress in the pressed material (dyn/cm²)
$\bar{\bar{\Phi}}_c$ = the tensor of the deformation stresses of the solid phase (dyn/cm²)
$\bar{\bar{\Phi}}_f$ = the tensor of the apparent stresses of the liquid phase (dyn/cm²)
$\bar{\bar{\Phi}}_s$ = the tensor of stresses transferred by the solid phase (dyn/cm²)
μ = dynamic viscosity of the liquid phase (dyn sec/cm²)
ϱ_f = true density of the liquid phase (gram/cm³)
ϱ_s = true density of the solid phase (gram/cm³)
$\sigma_x, \sigma_y, \sigma_z$ = the normal stress components of the tensor $\bar{\bar{\Phi}}_c$ (dyn/cm²)
$\tau_{xy}, \tau_{xz}, \tau_{yz} \ldots$ = the tangential stress components of the tensor $\bar{\bar{\Phi}}_c$ (dyn/cm²)

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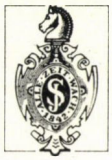

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CHANGES IN THE CARBOHYDRATE AND ORGANIC ACID CONTENTS OF TOMATOES AS A FUNCTION OF RADIATION DOSE AND STORAGE TIME

E. Kovács

(Received May 28, 1972)

Tomatoes (variety Original Frührot) in different stages of development were examined for carbohydrate and organic acid content by the method of Sweeley *et al.* (1963).

Changes in the carbohydrate and organic acid content of half-ripe and ripe tomatoes were determined after treatment with different radiation doses (5, 15, 30, 60, 250 and 500 krad).

A Hewlett—Packard 5750 gas chromatograph with a flame ionization detector was used for the measurements.

Fructose, glucose, saccharose and malic acid could be detected in the tomato samples.

The saccharose and malic acid contents of half-ripe tomatoes decreased as a result of irradiation with doses above 250 krad.

In ripe tomatoes, the malic acid content fell most markedly under the influence of irradiation.

The sugar content tended to decrease as a function of storage time, except for samples treated with 15 krad which usually delayed decomposition of the components tested.

The effect of irradiation was more extensive in half-ripe tomatoes than in the ripe ones.

A method for the simultaneous determination by gas chromatography of soluble carbohydrates and organic acids was elaborated by Sweeley *et al.* (1963).

The principle of the method is that the functional groups of sugars and organic acids (Rumpf, 1969) form trimethylethers (Davison & Young, 1969; Geyer, 1965; Partridge & Weiss, 1970) under given conditions (with trimethylchlorosilyl and hexamethyldisilazane).

Apart from carbohydrates, amino acids, peptides, steroids, flavonoids and acids of the Krebs-cycle can also be demonstrated by this method (Klebe *et al.*, 1966).

In this institute, the method was used for the quantitative determination of the carbohydrate and organic acid contents of the tomato fruit and an attempt was made to follow up the changes in these components under the influence of irradiation.

1. Materials and methods

1.1. Raw material and radiation treatment

Half-ripe and ripe tomatoes (variety Original Frührot) were used in the experiments. The degree of ripeness was judged subjectively, on the basis of colour.

Radiation treatment was carried out with the linear electron accelerator of the Institute for Radiation Technology, Nuclear Research Centre, Leopoldshafen, FRG, at dose levels of 5, 15, 30, 60, 250 and 500 krad. In the dose range of 5—60 krad, the samples were exposed to 5 krad by a single passage before the radiation source, whereas in the dose range of 250—500 krad to 50 krad under the same conditions.

The samples were stored at 10°C and 80—88% relative humidity (RH).

1.2. Description of the method

250 g tomatoes were homogenized in 250 ml extracting solution (methanol : water, 9 : 1), in a Turmix homogenizer. The extracting solution contained trehalose (400 mg/100 ml) as an inner standard.

Samples homogenized for 2 minutes were allowed to stand for 2—3 hours, centrifuged at 3 000 *g* for 5 min, and 2 ml amounts of the supernatant were placed in 2.5 ml Erlenmeyer flasks with ground glass stoppers. The contents of the flasks were dried by evaporation in a vacuum exsiccator, using P_2O_5 (in about 24 hours).

To the dry samples was added the reagent described by RUMPF (1969) (0.25 ml trimethylchlorosilyl, 0.50 ml hexamethyldisilazane, 1.25 ml pyridine).

1.3. Gas chromatograph

Analyses were carried out with a Hewlett—Packard 5750 gas chromatograph, using a flame ionization detector.

The separating column was a steel tube of 183 cm length, with an inner diameter of 0.5 cm.

Column packing: 10% VCCW 98 *Diatoport* 80—100 Mesh (Hewlett—Packard).

Carrier gas: helium.

Velocity of carrier gas: 30 ml · min^{-1}; 5 att pressure at exit.

Temperature of column: 150—300°C, programmed 10°C · min^{-1}.

Temperature of injection port: 260°C.

Temperature of flame ionization detector: 300°C.

Flow rate of hydrogen: 500 ml · min^{-1}.

The results were evaluated by the method of KAISER (1965) and expressed as % (weight/weight).

2. Results and conclusions

Sensitivity of the method can be read from the calibration curves (Fig. 1).

Fructose, glucose, saccharose and malic acid were demonstrated in tomato fruits in both stages of ripeness (Figs. 2—5).

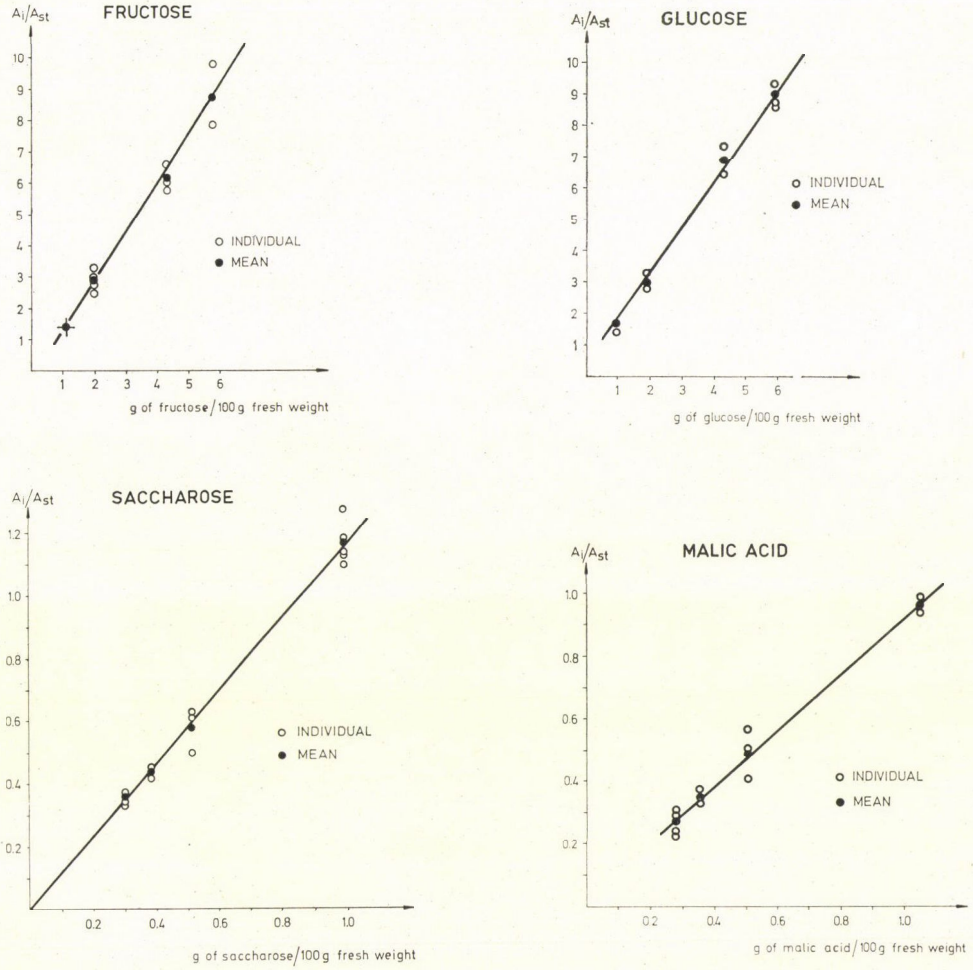

Fig. 1. Calibration curves for fructose, glucose, saccharose and malic acid. A_i: area of gas chromatographic peak corresponding to the examined carbohydrate; A_{st}: quantity of substance corresponding to trehalose; m_i: quantity of material tested, g; m_{st}: quantity of added trehalose, g; F: standard correction factor; r = correlation coefficient. Results of correlation calculations: Fructose: $A_i/A_{st} = -0.1560 + 1.5270 \cdot m_i/m_{st}$; $m_i/m_{st} = -0.1663 + 0.6404 \cdot A_i/A_{st}$; $r = 0.9889$; $F = 0.64$. Glucose: $A_i/A_{st} = 0.0577 + 1.5368 \cdot m_i/m_{st}$; $m_i/m_{st} = 0.0234 + 0.6477 \cdot A_i/A_{st}$; $r = 0.9977$; $F = 0.65$. Saccharose: $A_i/A_{st} = -0.0152 + 1.1957 \cdot m_i/m_{st}$; $m_i/m_{st} = 0.023 + 0.8220 \cdot A_i/A_{st}$; $r = 0.9914$; $F = 0.82$. Malic acid: $A_i/A_{st} = 0.023 + 0.9071 \cdot m_i/m_{st}$; $m_i/m_{st} = -0.017 + 1.0866 \cdot A_i/A_{st}$; $r = 0.9928$; $F = 1.09$

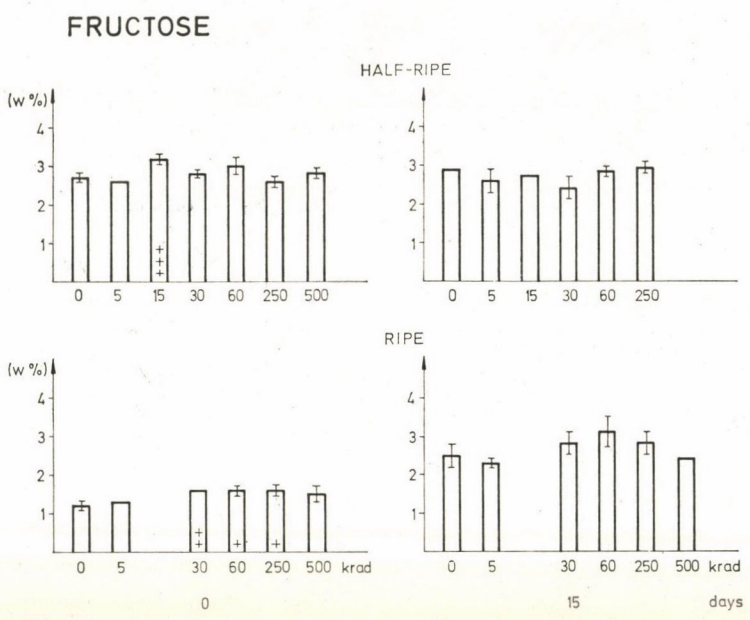

Fig. 2. Changes in the fructose content of tomatoes at different stages of ripeness as a function of radiation dose and storage time (10°C, 60—88% RH). w%: g of fructose/100 g fresh weight. The height of the histograms shows the mean value, vertical lines show the standard deviation (± s). Crosses (+, ++, +++) indicate levels of significance (P ≥ 95, 99, 99.9%) at which the fructose contents of treated tomatoes were higher than those of the untreated controls

2.1. Fructose

The fructose content of the half-ripe tomato increased under the influence of treatment with 15 krad. Difference from the untreated control was significant at the 99.9% probability level.

The fructose content of ripe tomatoes increased immediately after exposure to 30, 60 and 250 krad. The difference from the control was significant at the 99% level with 30 krad and at 95% with 60 and 250 krad.

The fructose content of half-ripe tomatoes scarcely changed during storage, whereas that of ripe tomatoes increased considerably.

Data of dose-response relationships can be seen from Table 1.

2.2. Glucose

The glucose content of the half-ripe tomato increased under the influence of 5 and 30 krad doses (Fig. 3) very highly significantly (P ≥ 99.9%), compared to the control.

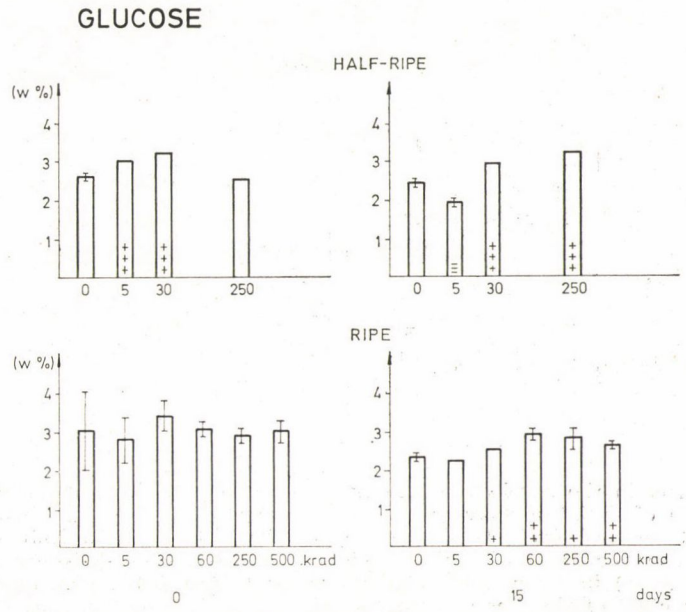

Fig. 3. Changes in the glucose content of tomatoes at different stages of ripeness a a function of radiation dose and storage time (10°C, 80—88% RH). w%: g of glucose 100 g fresh weight. The heights of the histograms indicate mean values, vertical lines the standard deviation ($\pm$ s). Crosses (+, ++, +++) and minus signs (−, −−, −−−) indicate levels of significance (P $\geq$ 95, 99, 99.9%) at which the glucose content of the treated samples was higher or lower, respectively, than that of the control

In untreated samples, the glucose content tended to decrease with storage and was significantly (P $\geq$ 99.9%) lower than that of the samples treated with 5, 30 and 250 krad, respectively.

Irradiation caused no measurable change in the glucose content of the ripe tomato, it decreased, however, in all the samples during storage, especially in the untreated samples. Thus the 0-krad samples contained significantly less glucose than those treated with 30 and 250 krad (P $\geq$ 95% and 99%), respectively.

Dose-response relationships can be seen in Table 2.

2.3. Saccharose

The saccharose content of half-ripe tomatoes was significantly increased by irradiation in the dose range between 5 and 60 krad (P $\geq$ 99.9%) and at 30 krad (P $\geq$ 99.0%) as well.

On the 5th day of storage, the saccharose content of the treated samples was found to have decreased significantly (P $\geq$ 99.9%) compared to that of the control.

Dose-response relationships are shown in Table 3.

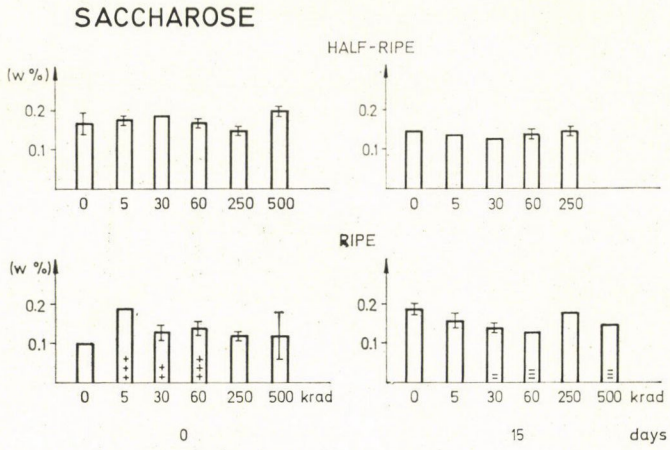

Fig. 4. Changes in the saccharose content of tomatoes at different stages of ripeness as a function of radiation dose and storage time (10°C, 80—88% RH). w% = g of saccharose/100 g fresh weight. The heights of the histograms indicate mean values, the vertical lines the standard deviation (± s). Crosses (+, ++, +++) and minus signs (−, −−, −−−) indicate levels of significance (P ≥ 95, 99, 99.9%) at which the saccharose content of the treated samples was higher or lower, respectively, than that of the control

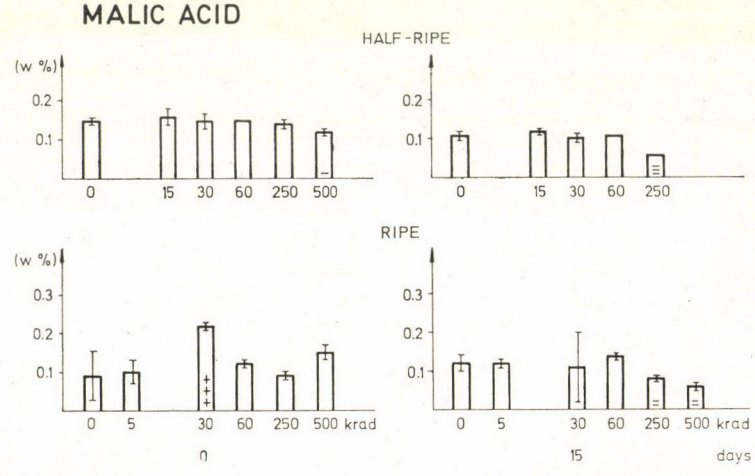

Fig. 5. Changes in the malic acid content of tomatoes at different stages of ripeness as a function of radiation dose and storage time (10°C, 80—88% RH). w%: g of malic acid/100 g fresh weight. Crosses (+, ++, +++) and minus signs (−, −−, −−−) indicate levels of significance (P ≥ 95, 90, 99.9%) at which the malic acid content of the treated samples was higher or lower, respectively, than that of the control

Table 1

Extent of radiation-induced changes in the fructose content of tomatoes as a function of the stage of ripeness, post-irradiation storage time and radiation dose

Treatments compared	Significance of difference between the effects of dose levels											
	Storage time (days)											
	0						15					
	Radiation dose (krad)											
I / II	0	5	15	30	60	250	0	5	15	30	60	250
krad	Half-ripe											
5	0						0					
15	+++	++					0	n				
30	0	+	−−				0	0	0			
60	0	n	0	0			0	0	0	0		
250	0	0	−−	−	−−		0	0	+	0	0	
500	0	0	−−	n	0	0	n	n	n	n	n	n
krad	Ripe											
5	0						0					
15	n	n					n	n				
30	++	+++	n				0	+	n			
60	+	++	n	0			0	+	n	0		
250	+	+	n	0	0		0	+	n	0	0	
500	0	0	n	0	0	0	0	++	n	—	—	0

Symbols used:

+: I < II
−: I > II
0: difference not significant
n: not tested
+ or −: difference significant at 95% probability level
++ or −−: difference significant at 99% probability level
+++ or −−−: difference significant at 99.9% probability level

Table 2

Extent of radiation-induced changes in glucose content of tomatoes as a function of the stage of ripeness, post-irradiation storage time and radiation dose

Treatments compared	Significance of difference between the effects of dose levels											
	Storage time (days)											
	0						15					
	Radiation dose (krad)											
I / II	0	5	15	30	60	250	0	5	15	30	60	250
krad	Half-ripe											
5	+++						−−−					
15	n	n					n	n				
30	+++	0	n				+++	+++	−−−			
60	n	n	n	n			n	n	n	n		
250	0	−−−	n	0	n		+++	+++	−−−	0	−−	
500	n	n	n	n	n	n	n	n	n	n	n	n
krad	Ripe											
5	0						0					
15	n	0					n	n				
30	0	0	0				+	+++	n			
60	0	0	0	0			++	+++	n	+		
250	0	0	0	0	0		+	0	n	0	0	
500	0	0	0	0	0	0	++	+++	n	0	0	0

Symbols used: see Table 1

Table 3

Extent of radiation-induced changes in saccharose content of tomatoes as a function of the degree of ripeness, post-irradiation storage time and radiation dose

Treatments compared	Significance of difference between the effects of dose levels											
	Storage time (days)											
	0						15					
	Radiation dose (krad)											
I / II	0	5	15	30	60	250	0	5	15	30	60	250
krad	Half-ripe											
5	0						0					
15	n	n					n					
30	0	0	n				0	—	n			
60	0	0	n	0			0	0	n	0		
250	0	———	n	0	——		0	0	n	+	0	
500	0	+	n	0	++	+++	n	n	n	0	n	n
krad	Ripe											
5	+++						0					
15	n	n					n	n				
30	++	——	n				——	0	n			
60	+++	——	n	0			———	—	n	0		
250	0	——	n	0	—		0	0	n	++	+++	
500	0	0	n	0	0	0	———	0	n	0	0	———

Symbols used: see Table 1

Table 4

Extent of radiation-induced changes in malic acid content of tomatoes as a function of the stage of ripeness, post-irradiation storage time and radiation dose

Treatments compared	Significance of difference between the effects of dose levels											
	Storage time (days)											
	0						15					
	Radiation dose (krad)											
I / II	0	5	15	30	60	250	0	5	15	30	60	250
krad	Half-ripe											
5	n						n					
15	0	n					0	n				
30	0	n	0				0	n	−−			
60	0	n	0	0			0	n	0	++		
250	0	n	0	0	−		−−−	n	−−−	−−	−−−	
500	−	n	−	0	−−	−	n	n	n	n	n	n
krad	Ripe											
5	0						0					
15	n	n					n	n				
30	+++	+++	n				0	0	n			
60	0	0	n	−−−			0	0	n	0		
250	0	0	n	−−−	−		−−	−−	n	0	−−	
500	0	+	n	−	0	++	−−	−−−	n	0	−−−	−

Symbols used: see Table 1

2.4. Malic acid

The malic acid content of the half-ripe tomatoes tended to decrease as a function of rising radiation dose, as well as a function of storage time; the decrease was very highly significant at 250 krad ($P \geq 99.9\%$).

No radiation doses except 30 krad caused a measurable change in the malic acid content of ripe tomatoes; in samples treated with 30 krad, it increased in a highly significant degree ($P \geq 99.9\%$). According to measurements on the 16th day of storage, the malic acid content fell to a highly significant extent ($P \geq 99.9\%$) in samples treated with doses above 250 krad, in comparison to the controls.

Dose-response relationships are shown in Table 4.

*

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EFFECT OF IONIZING RADIATIONS ON THE SHELF-LIFE OF TOMATOES AS A FUNCTION OF THEIR RIPENESS

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(Received May 28, 1972)

San Marzano tomatoes in three different stages of ripening were treated with 1, 4, 16, 64 and 256 krad doses of gamma rays. Storage temperature was 6—8°C, relative humidity 75—85%.

Radiation treatment of green and red tomatoes is inexpedient, because the former do not meet the organoleptic requirements while the storage life of the latter is not substantially extended.

The spoilage of pink-coloured tomatoes could be delayed by radiation doses of 4, 16 and 64 krad resp., but higher doses had an unfavourable effect.

The rate of weight loss of tomatoes in different stages of maturity can be reduced by irradiation.

Radiation treatment softened the texture of the tomato as an immediate effect, but during further storage, this effect diminished.

Irradiation of tomato fruits in the pink-coloured stage of ripeness seems to be promising.

Tomato is one of the horticultural products of low keeping quality. The search for suitable methods to prolong the storage life of tomatoes is therefore of great interest.

Preservation by ionizing radiations is one of the possible approaches. According to STADEN (1966), only low doses — 20 to 250 krad — are advantageous for the unripe tomato, because these do not stop post-harvest ripening, only delay it by 5—6 days. Irradiation of the green tomato fruit seems to be pointless, because at that stage of development gamma rays were found to accelerate spoilage of the fruit and formation of black spots (KOVALSKAYA *et al.*, 1962).

MAXIE *et al.* (1966) reported failure of radiation preservation of green- and yellow-coloured tomatoes. The shelf-life of pink-coloured tomatoes and of those ready for harvest can be prolonged by 4—12 days by irradiation with 200—350 krad. The organoleptic quality (smell, texture) of such tomatoes is superior to that of ripe tomatoes several days after harvesting.

Radiation treatment alters physiological processes in the tomato, and the efficiency of this treatment depends on the stage of ripeness at exposure.

1. Materials and methods

1.1. Test material

Tomatoes of the San Marzano variety were used throughout. They were procured from the Experimental Farm of the National Institute for Variety Testing, Tordas. Tomato fruits in three different stages of ripening were picked simultaneously.

1.2. Radiation treatment

The ^{60}Co-radiation source of 65 kCi capacity of the Radioisotope Institute of the Hungarian Academy of Sciences was used. The applied dose levels were 4, 16, 64, 128 and 256 krad, respectively. Dose rate was 20 and 100 krad $\cdot$ h^{-1}, respectively.

1.3. Storage

Irradiated samples were stored in the refrigerator at 6—8°C and 75—85% relative humidity.

1.4. Texture measurements

Two different instruments were used for this purpose, a penetrometer and a "Texturometer", produced by the Zenken Co. (Japan) on the basis of a license from General Foods Corp. (USA). The working principle of the instrument and the procedure of measurement were the same as described in an earlier paper (KOVÁCS & VAS, 1970).

Texture was measured individually on a total of 950 tomato fruits, on 2—5 spots on each fruit. Thus cca. 4 000 measurements were carried out.

Texture determinations with the penetrometer were performed on 20 individual fruits at each dose level. A Labor 5264 "Automatic" penetrometer was used for the purpose.

This instrument records automatically the degree of penetration at right angle to the surface (in 0.1 mm units) of a conventional cone of 150 $\pm$ 0.05 g total load (body length 44.6 mm, degree 30°C, height 15.2 mm) into the fruit in 5 sec, at 25°C. Averages of the penetrometer measurements were calculated for each dose level. The effect of the applied radiation doses was evaluated by analysis of variance.

The Texturometer was used to determine the hardness of the fruit (KOVÁCS & VAS, 1970).

All experiments were carried out under predetermined conditions.

Characteristics of the fruit:

Tomatoes of identical size, cut into two halves (measurements were made on the skin-covered side). The thickness of the half-fruit was 15 mm at its highest point.

Characteristics of the Texturometer:

— a bronze plunger, shaped like a dental plate,

— a mechanical head (chewing device), operative at the rate of 12 bites per min,

— a recording paper strip moving at the rate of 750 mm per min,

— receiver for the electric signal, operative in the range of 0—2.5 V (at 0.5 V), at 1 Attenuator 1 adjustment.

The results were evaluated by analysis of variance.

1.5. Measurements of weight

Weights of the same 10×5 tomato fruits per dose level were determined, at an accuracy of ± 50 mg. Percentage weight loss was calculated from the weight measurements, and evaluated by mathematical-statistical analysis. Linear regression was calculated (WEBER, 1961) and regression lines for each dose level were compared by Student's t test, on the basis of the b values. For comparison, the data were formulated mathematically, as proposed by SVÁB (1967).

1.6. Examination of spoilage

Two hundred ($N = 200$) tomatoes were examined at each dose level (a total of 3 000), as a function of storage time.

Spoilage was expressed as percent of the samples tested. The extension of the keeping time was assessed graphically, on the basis of the time elapsing until 10 or 20% spoilage (VAS, 1959, 1971).

2. Results

2.1. Progress of spoilage

Prolongation of keeping time relative to that of the control sample at spoilage levels of 10 and 20% is shown in Fig. 1.

At the 10% level of spoilage, the extension of keeping time of tomatoes in the green state was most favourable (about 27%) when irradiated with 4 krad. The keeping time of pink tomatoes was extended by about 8% (4, 16 and

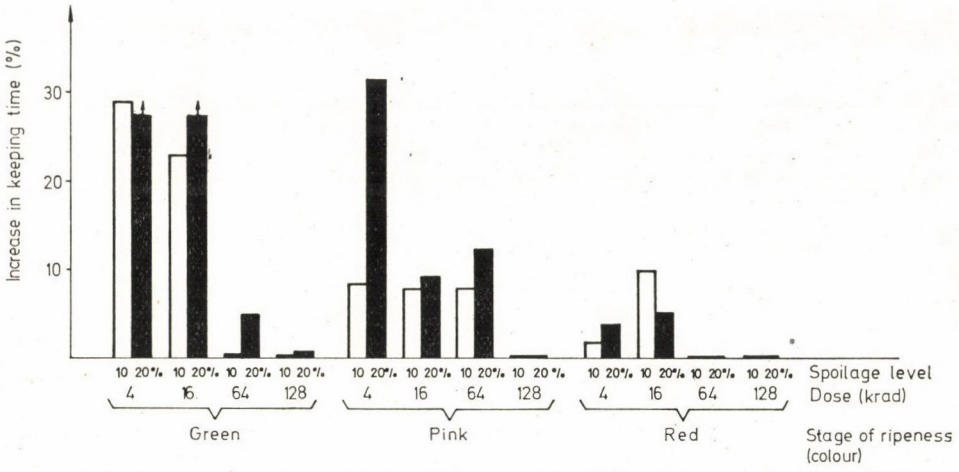

Fig. 1. Relative keeping times (at 6—8°C, 75—85% RH) of tomato fruits at different stages of ripening as a function of the radiation dose, based on the number of days elapsing until 10 or 20% spoilage

64 krad) but, with red tomatoes, irradiation did not noticeably extend shelf-life. No extension of the storage time could be achieved by treatment with 128 krad. At the spoilage level of 20%, storability of green tomatoes could be extended by about 26% by treatments with 4 or 16 krad. For pink tomatoes, the 4 krad dose proved to be optimal, prolonging keeping time by about 31%. Effect on red tomatoes was the same as above.

2.2. Weight loss

Weight loss during storage should also be taken into consideration. Effects of irradiation on weight are shown in Fig. 2.

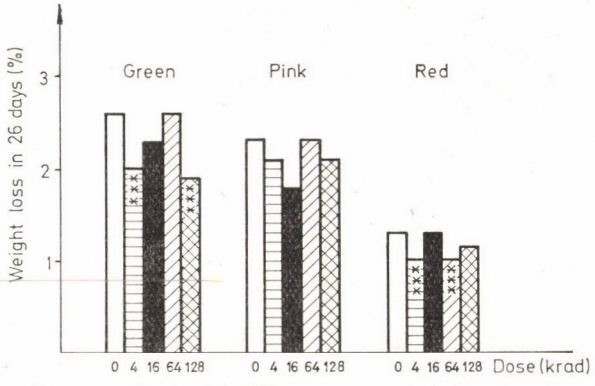

Fig. 2. Weight loss of tomato fruits in different stages of ripening as a function of the radiation dose (storage at 6—8°C, 75—85% RH, N = 40). Weight loss in the irradiated sample was very highly significantly ($P \geq 99.9\%$) lower than that in the control

The weight loss in green tomatoes was lowest after treatment with 4 or 128 krad, in pink tomatoes with 16 krad. For red tomatoes, doses of 4 and 64 krad were optimal. On considering a 26-day storage period relative weight loss percentages are the following: 30% for green tomatoes, 24% for pink tomatoes and 22 and 26% for red tomatoes on treatment with 4 and 64 krad, respectively.

2.3. Texture changes

In the examined three stages of ripeness, the texture of tomatoes can be characterized as follows:

Average penetrometer values for green tomatoes differed significantly at the three different periods of storage (0, 9 and 22 days, resp.).

On 0 day, the texture of the samples exposed to 64 and 256 krad was significantly ($P \geq 99.9\%$) inferior to that of the control fruits, while samples exposed to the other dose levels did not significantly differ from the latter.

After 9 days, the sample treated with 256 krad was significantly ($P \geq 99.9\%$) inferior to the rest.

256 krad effected in fact a permanent softening of the texture, while softening caused by 64 krad diminished after a certain time of storage, or at least the texture of the tomatoes thus treated did not differ from that of the other samples, ripened during the same period. Treatment with 4 krad had no notable influence on the texture of green tomatoes.

Effects of irradiation on the texture of pink tomatoes were as follows:

Measurements performed immediately after radiation treatment on 0 day showed the texture of the sample irradiated with 256 krad to be significantly ($P \geq 99.9\%$) inferior to that of the rest, and the case was the same after 10 days of storage ($P \geq 99.9\%$).

On the 28th day, the sample treated with 64 krad was significantly ($P \geq 99\%$) inferior to the other samples as regards texture (Table 2).

The texture of red tomatoes was significantly softened by irradiation at all dose levels tested. Difference from the control reached the 99.9% probability level even in the case of fruits treated with 64 krad.

After 8 days of storage, the texture of the sample treated with 1 krad was significantly superior to that of the control, while the other irradiated samples did not significantly differ from one another in respect of texture (Table 3).

Texture was also measured with the Texturometer. Regression coefficients calculated from penetrometer and Texturometer data are shown in Fig. 3.

The correlation coefficient 0.7 clearly indicates that the texture values assessed by two different instruments correlated not too closely. (With both instruments simultaneously picked individual fruits of the same lot, but not identical fruits, were measured.)

Table 1

Effect of ionizing radiations on the texture of tomatoes, expressed in mean penetrometer degrees (0.1 mm), as a function of radiation dose and storage time
Unripe, green-coloured tomatoes

Dose (krad)	0 day		9th day		22nd day	
	Average x_i	Difference $(x_o - x_i)$	Average x_i	Difference $(x_o - x_i)$	Average x_i	Difference $(x_o - x_i)$
0	7.660	—	15.569	—	25.234	—
1	7.691	−0.031	29.881	−14.312***	20.755	4.479
4	8.701	−1.041	16.024	−0.455	23.983	1.251
64	13.035	−5.375***	17.224	−1.655	30.004	−4.770
256	20.958	−16.298***	19.857	−4.288***	38.043	−12.809
Significance of difference	$LSD_{95\%} = 1.242$ $LSD_{99\%} = 1.634$ $LSD_{99.9\%} = 2.092$		$LSD_{95\%} = 2.092$ $LSD_{99\%} = 2.754$ $LSD_{99.9\%} = 3.525$		$LSD_{95\%} = 5.755$ $LSD_{99\%} = 7.574$ $LSD_{99.9\%} = 9.695$	

Symbols used:
LSD = least significant difference
— = not differing from the control
* = differing from the control at 95% probability level
** = differing from the control at 99% probability level
*** = differing from the control at 99.9% probability level
x_o = mean for untreated sample
x_i = mean for radiation-treated sample

Table 2

Effect of ionizing radiations on the texture of tomatoes, expressed in mean penetrometer degrees (0.1 mm), as a function of radiation dose and storage time
Half-ripe, pink-coloured tomatoes

Dose (krad)	0 day		10th day		26th day	
	Average x_i	Difference $(x_o - x_i)$	Average x_i	Difference $(x_o - x_i)$	Average x_i	Difference $(x_o - x_i)$
0	34.561	—	62.296	—	85.534	—
1	32.098	2.463	62.735	−0.439	82.699	2.835
4	37.059	−2.498	64.0919	−1.723	91.307	−5.773
64	38.749	−4.188	64.596	−2.300	97.845	−12.311**
256	47.549	−12.988***	71.510	−9.214**	88.952	−3.418
Significance of difference	$LSD_{95\%} = 4.341$ $LSD_{99\%} = 5.713$ $LSD_{99.9\%} = 7.312$		$LSD_{95\%} = 5.521$ $LSD_{99\%} = 7.266$ $LSD_{99.9\%} = 9.301$		$LSD_{95\%} = 8.911$ $LSD_{99\%} = 11.727$ $LSD_{99.9\%} = 15.010$	

For symbols used: see Table 1

Table 3

Effect of ionizing radiation on the texture of tomatoes, expressed in mean penetrometer degrees (0.1 mm), as a function of radiation dose and storage time

Ripe, red-coloured tomatoes

Dose (krad)	1st day		8th day	
	Average x_i	Difference $(x_o - x_i)$	Average x_i	Difference $(x_o - x_i)$
0	51.632	—	71.875	—
1	60.248	—8.616**	61.759	10.166***
4	57.691	—6.059*	69.965	1.910
64	63.855	—12.223***	67.024	4.851
256	73.518	—21.886***	74.464	—2.589
Significance of differences	$LSD_{95\%} = 5.580$ $LSD_{99\%} = 7.344$ $LSD_{99.9\%} = 9.400$		$LSD_{95\%} = 5.477$ $LSD_{99\%} = 7.208$ $LSD_{99.9\%} = 9.226$	

For symbols used: see Table 1

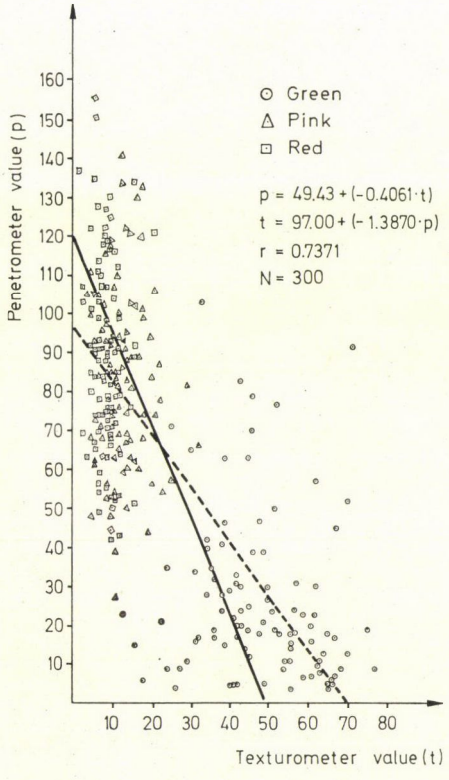

Fig. 3. Correlation analysis of texture data obtained by measurements with penetrometer and Texturometer (r = correlation coefficient)

3. Conclusions

On the basis of spoilage studies, the radiation treatment of tomatoes picked in the pink state seems to be promising. STADEN (1966) concluded from measurements on tomato fruits in different stages of maturity that radiation doses of 20—250 krad can only prolong the shelf-life of pink tomatoes. Attempts to extend the storage life of red ripe tomatoes failed also in our hands.

According to FRUMKIN and PETRASH (1965), doses of 200 or 400 krad effectively prolong the keeping time of tomatoes by destroying their surface microflora, above all some highly sensitive molds. KOVALSKAYA *et al.* (1962) reported that irradiated red tomatoes were not inferior in quality to untreated ones, and their microbiological spoilage began 5—10 days later at 20—22°C.

It is generally known that radiation treatment causes a temporary or permanent softening in most fruits and vegetables (MAXIE *et al.*, 1966; MASSEY *et al.*, 1964; KOVÁCS & VAS, 1970).

The influence of irradiation on the texture of tomato depended on the latter's stage of ripeness. Softening was temporary with pink and red tomatoes, but permanent with green tomatoes.

Softening of texture consequent to radiation treatment may be important from the point of view of industrial processing (*e.g.* in respect of improving juice yield or peeling capacity).

Notable is the effect of low radiation doses (1—16 krad). Since their effect does not result from destruction of the microbial flora, it is probably achieved by increasing the resistance of the fruit in some hitherto unknown way. The growth stimulating effect of low doses has been proven by several authors (FLAIG & SCHMID, 1966; SIMONIS, 1966).

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PERSPECTIVES OF THE RADURIZATION OF MEAT PRODUCTS

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At present radiation doses of 0.2—0.6 Mrad are the most promising for meat treatment as they do not result in undesirable changes of the organoleptic properties of the products.

Radurization of the half-finished products beefsteak (I), "schnitzel" (II) and comminuted salted meat (III), vacuum packed in polymer packages, was studied.

In order to exclude exudation and inhibit oxidative changes some samples (II) were rolled in an ascorbic acid-tripolyphosphate mixture. Some samples (III) were cured with pyrophosphate.

Irradiation was carried out by means of gamma rays with doses of 0.2—0.4 (I), 0.6 (II) and 0.3 (III) Mrad. Samples were kept at +5°C.

On the basis of organoleptic and chemical investigations, microbiological and technological tests (for III) it was found that the shelf-life of high-quality beefsteak irradiated with doses of 0.4 Mrad lasted 4 weeks, with doses of 0.2 Mrad for 2 weeks, with 0.3 Mrad for 3 weeks. Schnitzel breaded and irradiated with 0.6 Mrad was of high quality after 2 months of storage. The meat cured with pyrophosphate kept its technological and consumer qualities during the same period of time.

It was calculated (PALMIN *et al.*, 1970) that by using a ^{60}Co installation, with an activity of 400 kg-equivalent of radium and irradiating a ton of half-finished products per day with doses of 0.4 Mrad and a coefficient of radiation utilization of 30%, transport expenses of radurized half-finished meat products (I) would be profitable at distances above 100 km as compared to the transport of cattle or, at distances over 700 km, as compared to that of carcasses in halves or quarters (Fig. 3).

Investigations in the last two decades have shown the extraordinary complexity of the problem of using ionizing radiation in meat processing. That is why, in spite of extreme efforts made in the direction of using the irradiation method for meat processing, its application has not yet become a reality. The difficulty of the problem lies in the fact that meat components may be subject to changes under the influence of radiation which changes depend on the permanently varying conditions which prevail in the tissues (pre-slaughter conditions, post-mortem period, displacements in enzyme systems, and in the redox potential, in pH, changes in composition, etc.)

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and on external conditions (microbial contamination, partial pressure of gases, temperature, radiation dose, etc.). Radiation changes of tissue components and accumulation of some products of destruction or modification are the causes of the well-known caution in the estimation of their technological and biological significance.

At present radiation doses of 0.2—0.6 Mrad are the most promising for meat treatment as they do not result in undesirable changes of the organoleptic properties of the products.

Vacuum packaging or antioxidants are successfully applied to eliminate or reduce oxidative changes of lipids. In order to inhibit the action of proteolytic enzymes some attempts have been made at utilizing chemicals (DRAKE *et al.*, 1957; FRUMKIN *et al.*, 1962), pH changes (NIEWIAROWICZ & PALMIN, 1964), pre-slaughter adrenalization (RADOUCO-THOMAS *et al.*, 1959; PALMIN, 1964) and thermal treatment (DOZORETS *et al.*, 1960), though in the latter case ready-to-cook meat is produced. But not all the methods mentioned above are sufficiently effective and convenient in all cases of radurization.

Radurization of half-finished beefsteak, "schnitzel" (fried cutlet) and comminuted salted meat, vacuum packed in plastic bags (viscothene, polyethylene-cellophane-PC) was studied.

Radurization of half-finished beef. Beef radurization at doses not reaching the threshold level was considered.

These doses would allow to maintain the quality of half-finished products on storage for 2—3 weeks at 5°C, whereby, in our opinion, the absence of flavour changes in the irradiated product and its normal appearance must be the criterion of acceptability of the selected dose and of the whole technology of radurization. Furthermore the chosen parameters of radiation treatment should be economically justified, *i.e.* capital invested in the construction of the corresponding irradiating installation must pay off within the time accepted in the meat industry.

In this connection our investigation contains technological research which would guarantee an adequate level of acceptability of flavour and market qualities of beef including its appearance.

Radurization of minced meat. Another aspect of the investigations consists in the study of possibilities to prolong storage life of sausage filling prepared from chilled meat for cooked sausage production.

Cooked sausages of the highest quality are known to be produced from chilled but not frozen meat.

However, the supply of the plants with cooled meat is associated with the delivery of cattle and restricted by seasonal prevalence.

In recent years great attention has been paid to the problem of the specialization of sausage production as a result of which it seems expedient

to deliver salted sausage meat or fillings to large cities. To solve this problem it is necessary to possess data on the storability of salted meat without losses in desirable qualities. In addition, the problem of a possible prolongation of the storage period arises as well.

Considering what had already been said we decided to study the physico-chemical changes taking place in the ageing process of salted meat used in the production of boiled sausages and frankfurters.

Storage life of corned beef depends on various factors, first of all on the development of the microflora and on physico-chemical and biochemical processes. In this connection it seemed interesting to investigate the advantages of some curing methods, special packaging and gamma radiation.

Radurization of half-finished pork products. To solve the problem of radurization of half-finished pork products, first of all the applicability of various film wrappings to packaging irradiated half-finished products was investigated, namely: cellophane, polyethylene, laminated polyethylene-cellophane film: PC-II (of home production) and three-layer polyethylene film and foil as well as cellophane (imported).

Out of the two films most suitable for the storage of half-finished products (PC-II and a three-layered specimen), we used the former in our subsequent work (because of its easier availability).

In previous work it has been established that gamma radiation treatment at a dose level of 0.6 Mrad allowed to keep half-finished pork products vacuum packed in the film PC-II without deterioration and to maintain their normal flavour for two months at +2 to +5°C (ANISIMOV *et al.*, 1966). Accumulation of exudate within the package, gradual discoloration of the meat surface, some oxidative changes in lipids were observed to take place during the storage life of irradiated half-finished products.

As a result of a number of experiments it was found that exudation might be greatly reduced by means of surface rolling of the samples prior to irradiation in a mixture containing sodium chloride, sodium tripolyphosphate (TPP) and ascorbic acid in quantities of 2%, 0.3% and 0.25%, respectively, as related to the weight of meat.

The introduction of sodium chloride and phosphates increases hydration of proteins, promotes the improvement of the water-binding capacity of meat and that of its consistency and, in addition, stabilizes the colour of irradiated pork at all stages of the experiments. Antioxidant properties of this mixture were shown simultaneously.

During prolonged storage of irradiated meat a reduction in quality of the product takes place due to the destruction of proteins and low molecular weight compounds, hydrolysis and oxidation of lipids.

The aim of our investigations was to study the changes occurring in pork lipids as a result of irradiation and subsequent storage.

1. Materials and methods

1.1. Radurization of half-finished beef products

For an objective judgement of meat quality stabilization achieved by treatment with different doses of ionizing radiation an experiment using the standard method of freshness determination was carried out to estimate quality preservation.

Besides organoleptic tests, the standard method includes a number of chemical analyses, quantitative determination of volatile fatty acids, reaction with copper sulphate and determination of amino-ammonia-nitrogen content. Bacteriological investigations are also included.

Fillet samples *(musculus psoas)* from 8 two-year-old bull-calves of the same breed were taken at the Moscow meat-packing plant. Every muscle was cut into 8 pieces nearly equal in weight, which were then packed individually in mylar-film bags in a vacuum packer. Samples prepared in this way were divided into 4 equal groups, each group including an equal amount of meat samples from every animal.

Groups of 20 samples were irradiated with different doses, namely 0.2 Mrad, 0.3 Mrad, 0.4 Mrad and 0.6 Mrad. One group of the samples was not irradiated and served as the control group. After irradiation all samples were put into a refrigerator, where they were kept for 4 weeks at 5°C.

Periodically, every 7 days four samples from each of the groups were taken for organoleptic analysis.

Both raw and ready-to-serve samples were analyzed.

Cooking treatment consisted in pan-frying in refined corn-oil, the pan being well pre-heated. Samples were considered to be ready-to-use by the clear juice flowing from a puncture made by a cook's needle.

The quality of the raw meat was estimated by odour, colour and appearance. Flavour, odour and consistency were analyzed to establish the quality of the fried product. The Geneva ten-score system was used to evaluate the individual characteristics.

1.2. Radurization of minced meat

One part of beef comminuted on the gyroscope was cured according to technological instructions, while to another part anhydrous sodium pyrophosphate (*PP:* $Na_4P_2O_7$) was added in the amount of 0.3%, in addition to salt, nitrate and nitrite. Then the meat was vacuum packed in PC in 100 g units at a residual pressure of 10.0 mm Hg or at atmospheric pressure. Part of the samples packed in either way were exposed to gamma radiation at a dose-level of 0.3 Mrad. The samples were kept at +4°C as follows: the non-irradiated meat for 30 days and the irradiated samples for 60—90 days.

pH was determined in aqueous extracts (1:10) by means of a potentiometer.

Water-holding capacity *(WHC)* was determined by the press-method of *Grau* and *Hamm* as modified by *Valovinskaya* and *Kelman*.

The degree of proteolysis was established by determination of amino-ammonia-nitrogen *(AN)* according to MOORE and STEIN (1968).

Acid number *(ACN)* and peroxide number *(PN)* were established by the modified method of VNIIMP (PIUL'SKAYA, 1958), while the thiobarbituric acid number *(TBA)* was determined by means of the method of TURNER and co-workers (1954).

In the course of the investigations the analysis of microbial contamination has been made. The experimental data obtained were compared with the organoleptic indices of samples of cooked sausage prepared from the same meat.

1.3. Radurization of half-finished pork products

Investigations were carried out on *longissimus dorsi* hog muscles 2 to 3 days after slaughter. Meat steaks in the form of "schnitzel" of about 125 g apiece were vacuum-packed in the film PC-II. Half of the samples were subjected to surface treatment by the above-mentioned mixture just before packaging. These samples were irradiated with gamma rays at a dose level of 0.6 Mrad and were kept at +5°C for two months.

PN, *ACN*, the amount of malonic aldehyde according to TURNER and co-workers (1954), the total amount, the qualitative and quantitative composition of volatile carbonyl compounds *(CC)*, the amount of total and individual volatile fatty acids *(VFA)* were determined in the samples before and after radiation as well as after a lapse of 20, 40, 60 days, respectively.

The extraction of volatile *CC* was carried out by vacuum-distillation in a current of argon (PIPPEN *et al.*, 1958). *CC* were transformed into 2—3 dinitrophenylhydrazones and then separated into individual components by ascending thin-layer chromatography (PALMIN & MAKAROVA, in press). The amount of individual *CC* was determined by means of elution of the spots from the plates. The total amount of volatile *CC* was calculated from the sum of the individual components.

VFA were extracted by means of steam distillation (KRYLOVA & LYASKOVSKAYA, 1965) and the composition and amount of the individual *VFA* fractions were investigated by gas-liquid chromatography at different stages of storage of the irradiated samples.

2. Results

2.1. Radurization of half-finished beef products

Control (non-irradiated) samples of beefsteaks (Fig. 1) had lost their acceptability by the end of the second week of exposure. By that time samples

irradiated with the lowest dose of 0.2 Mrad had been scored below 7 points with regard to the majority of indices.

Half-finished beef products irradiated with the dose of 0.3 Mrad exhibited a tendency to quality reduction only in the fourth week of storage. During that period meat irradiated with 0.2 Mrad lost its acceptability.

High quality (about 7.5—8 scores) was maintained up to the end of the experiment only by half-finished beef products irradiated with doses of 0.4 and 0.6 Mrad, respectively.

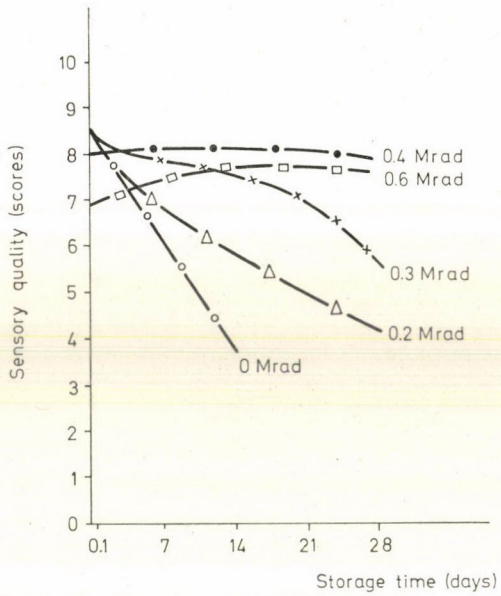

Fig. 1. Changes in sensory properties of irradiated half-finished beef products in the course of storage at 5°C

Some preference was given to the dose of 0.4 Mrad by the taste panel during all the experiments over the slightly dry consistency of the samples irradiated with 0.6 Mrad. Chemical investigations of meat freshness carried out by standard methods showed that radiation treatment with accepted doses caused but insignificant changes in the values of the indices studied.

The content of amino-ammonia-nitrogen *(AN)* increased in all test groups immediately after irradiation as compared to the control group, but this increase was slight and identical in all the groups.

A moderate increase in the value of this index was observed during a three-week refrigerated storage as a result of autolysis.

A dependence of the volatile fatty acid content *(VFA)* in meat on the dose of radiation was found immediately after radiation treatment and on

further storage. The higher the dose applied, the greater was the shift in this index towards higher values. Afterwards, during the first week of storage the *VFA* content decreased in the irradiated samples.

With doses higher than 0.2 Mrad a comparatively low *VFA* content was maintained up to the end of storage while in the control group and in samples irradiated with a dose of 0.2 Mrad there was a rapid increase in the index which showed the intensification of chemical breakdown processes under the influence of microorganisms.

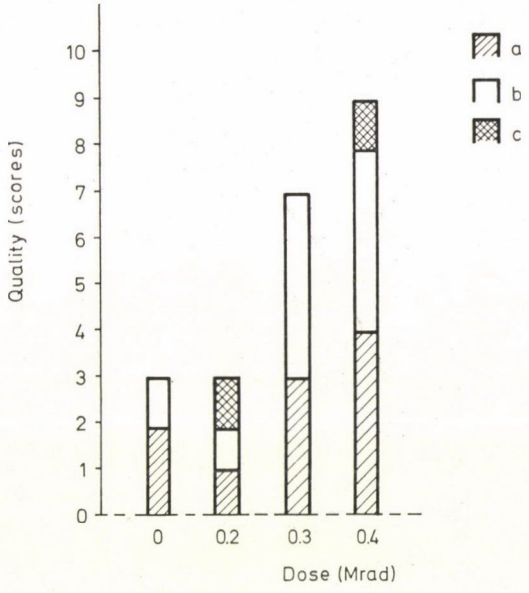

Fig. 2. Meat freshness on the 22nd day after irradiation according to standard chemical data. a — volatile fatty acids; b — reaction with $CuSO_4$; c — amino-ammonia-nitrogen

The comparison of summary quality scores (Fig. 2) obtained as a result of data processing of standard chemical methods for the determination of meat freshness degree (0.2 Mrad — 3 scores; 0.3 Mrad — 7 scores; 0.4 Mrad — 9 scores; the control — 3 scores) proves that the treatment of half-finished products with a dose of 0.4 Mrad is preferable if a 3-week storage at a temperature near to but above zero is applied.

The results of bacteriological analysis have confirmed this conclusion. In the samples of the control group the amount of microbial cells per one g of meat ranged from 1.3 to $1.5 \cdot 10^7$ on the 22nd day of storage, while in the samples irradiated with 0.2 Mrad it was between 1.5 to $3.0 \cdot 10^6$. With 0.3 Mrad

the amount of microbial cells varied between $6.5 \cdot 10^5$ and $7 \cdot 10^5$, and with 0.4 Mrad only 35—40 cells were found, that is 6 log cycles less than in the control sample.

It was found that the quality of radurized half-finished beef products depended to a great degree on the properties of the packaging material and on the method of packaging. No deviations from the normal were observed in biochemical changes related to autolysis and the dynamics of physico-chemical processes in radurized meat which had been vacuum packed or packed to fill entirely the packaging volume if the packaging materials had a low gas permeability.

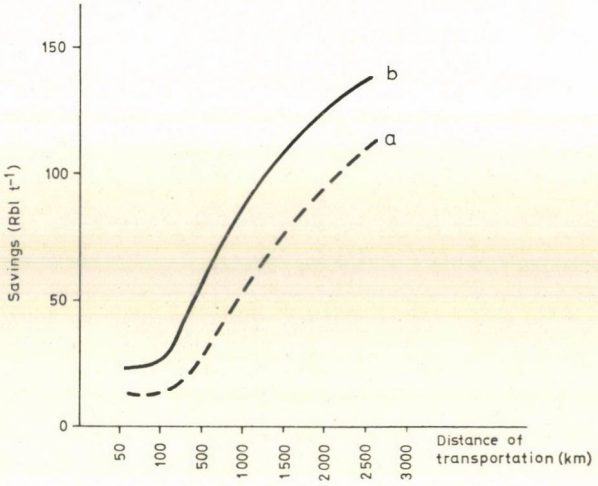

Fig. 3. Savings achieved by transporting half-finished products depending on the distance of transport as compared with the transport of: a — meat carcasses in halves and quarters; b — cattle

Radurization of natural half-finished meat products may be used at meat packing plants in areas abounding in raw material with the aim of transporting meat products to large cities and districts with poorly developed animal breeding.

It was calculated (PALMIN *et al.*, 1970) that by using a ^{60}Co installation, with an activity of 400 kg-equivalent of radium and irradiating a ton of half-finished products per day with doses of 0.4 Mrad and a coefficient of radiation utilization of 30%, transport expenses of radurized half-finished meat products (I) would be profitable at distances above 100 km as compared to the transport of cattle or at distances over 700 km as compared to that of carcasses in halves or quarters (Fig. 3).

2.2. Radurization of minced meat

The lowest pH value of 5.8 was that of the initial samples of non-irradiated meat.

During the storage period the pH increased. pH of meat packed under ordinary conditions reaches the range of 6.25—6.35 on the 20th day, pH of vacuum-packed meat changes slightly during the first ten days. By the end of the storage period its pH is by 0.2—0.3 units lower than that of meat

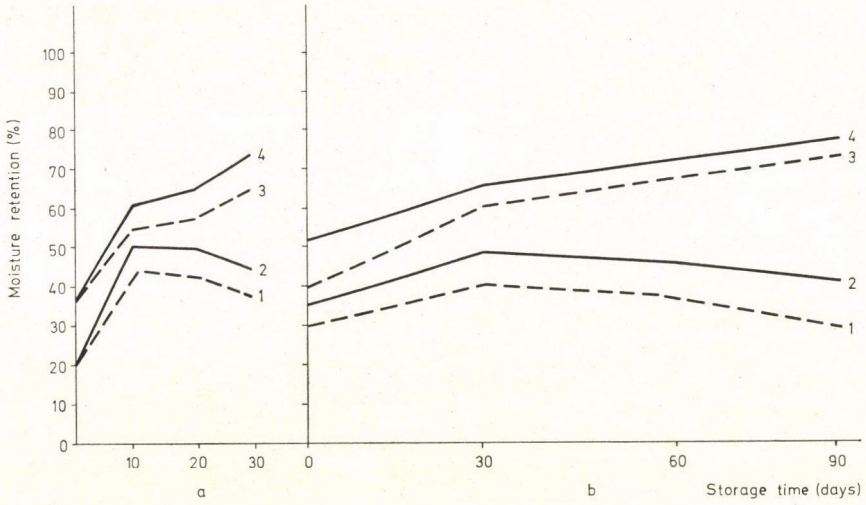

Fig. 4. Changes in moisture-retaining ability of cured meat during storage at 5°C. a — non-irradiated; b — irradiated; ——— wrapped in vacuum; - - - - - - wrapped in contact with air; 1, 2 — cured without pyrophosphate; 3, 4 — cured with pyrophosphate

packed under atmospheric pressure. The difference in pH caused by the addition of *PP* is maintained during the whole period of storage (irradiated and non-irradiated meat).

Radiation causes an insignificant increase in pH. Dynamics of *WHC* of the samples are different (Fig. 4). Meat containing *PP* showed higher values.

WHC noticeably increases during nearly the whole storage period and decreases only by the end of storage. Its value is as high as 65—72% in non-irradiated samples and about 72—75% in irradiated ones.

A gradual reduction in meat hydration was observed after 20 days of storage in non-irradiated samples salted without *PP*, nevertheless such meat had a high *WHC* even on the 30th day.

In all cases samples packed in vacuum had a higher *WHC*. On storage of irradiated as well as non-irradiated salted meat the *AN* content increases (Fig. 5). After 30 days *AN* ranges between 95 and 115 mg% in samples of non-irradiated meat, while in vacuum this value is significantly lower.

The addition of *PP* causes a slight rise in *AN* level. Later on this value is higher in salt meat stored according to the prevailing technology (in the presence of air).

At the beginning radiation produces a certain increase in *AN* content but later on the process of its formation is retarted. During 30 days the *AN* level in meat irradiated in vacuum is lower than in non-irradiated meat under identical packaging conditions, and is equalized only on the 60th day.

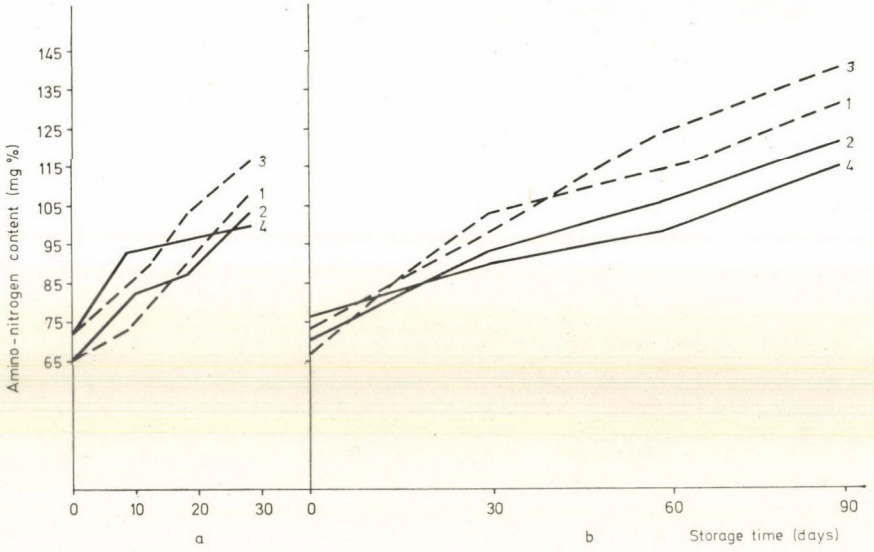

Fig. 5. Changes in amino-nitrogen content during storage, at 5°C, of cured meat. a — non-irradiated; b — irradiated; ——— wrapped in vacuum; - - - - - - wrapped in contact with air; 1, 2 — cured without pyrophosphate; 3, 4 — cured with pyrophosphate

The addition of *PP* (Fig. 5) causes retardation of processes related to *AN* accumulation in this case, too.

Fats are known to undergo oxidative changes during the process of storage (LÜCK & KÜHN, 1959). Oxidation proceeds more noticeably under the influence of gamma rays.

According to the data obtained the lowest values of *ACN*, *PN* and *TBA* were observed in the samples with added *PP* (0.07, 0.0015 and 5.25, respectively). The *ACN* in non-irradiated samples was within the range of the standard (up to 1.4) for 30 days.

There is a sharp change in the lipids of irradiated meat due to hydrolytic breakdown, but the addition of *PP* delays the development of that process and on the 60th day of storage *ACN* complies with the standard. Oxidative changes proceed in a different way. *PN* is also somewhat higher

in irradiated meat. *PP* addition in vacuum allows to reduce the increase in peroxides.

The peculiarity of the oxidative changes in lipids is expressed by *TBA* (Fig. 6). Irradiation is found to cause insignificant increase in *TBA*. On storage this index increases in both irradiated and non-irradiated meat, though less markedly in the latter. Thus, irradiation seems to retard the development of oxidative changes in this direction.

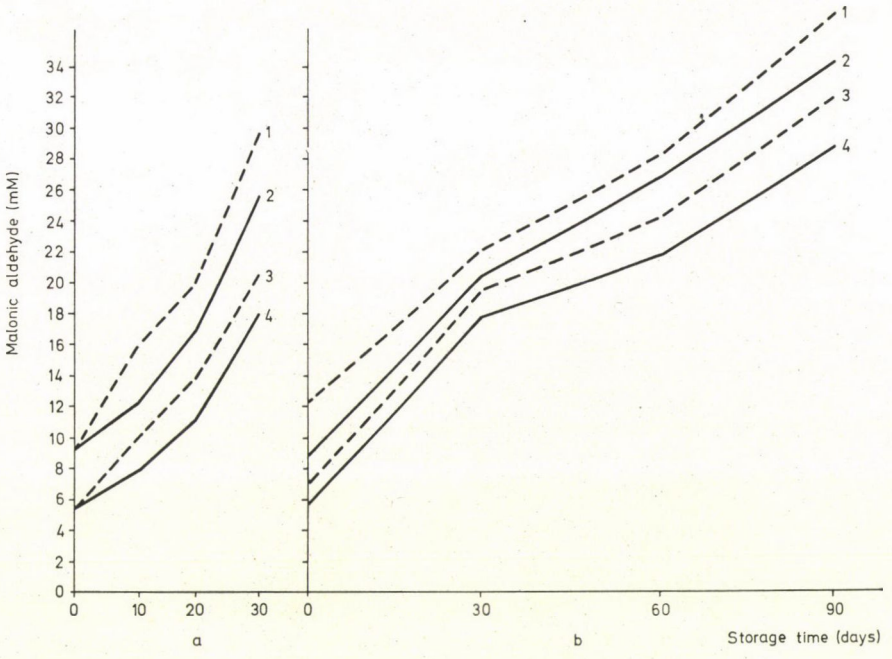

Fig. 6. Changes in *TBA* during storage of cured meat at 5°C. a — non-irradiated; b — irradiated; —— wrapped in vacuum; - - - - - - wrapped in contact with air; 1, 2 — cured without pyrophosphate; 3, 4 — cured with pyrophosphate

The microflora is known to play an important role in the process of cured meat storage, as besides oxidative changes product spoilage may be due to this factor.

Three factors influence the development of the microflora (Fig. 7): packaging, curing conditions and irradiation. Effective suppression of micro-organism growth takes place when packing is combined with the addition of *PP* on curing.

Contamination of such meat per gramme amounts only to $3.5 \cdot 10^3$ on the 30th day as compared to the meat without *PP* addition, where this value is $5.5 \cdot 10^3$.

Irradiation of meat results in a marked reduction of bacterial contamination (10—15 times) and is around $8.5 \cdot 10\ g^{-1}$. On further storage of such meat the microflora is developing very slowly. In the presence of *PP* bacterial contamination is much lower than in other samples and after a 2-month storage it amounts to $2.7 \cdot 10^2\ g^{-1}$, *i.e.* it is one third of that of the original non-irradiated meat ($9 \cdot 10^2\ g^{-1}$).

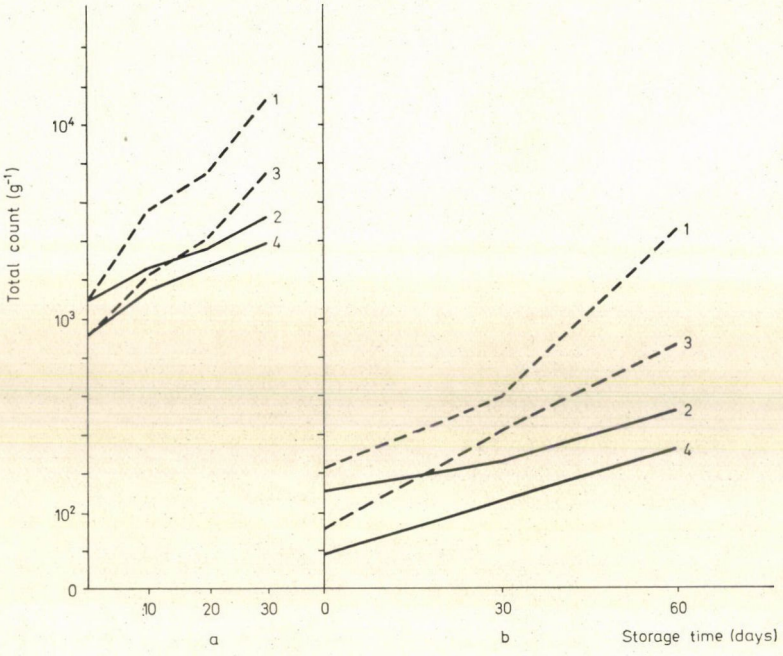

Fig. 7. Total microbial count of cured meat during storage at 5°C. a — non-irradiated b — irradiated; ——— wrapped in vacuum; - - - - - - wrapped in contact with air 1, 2 — cured without pyrophosphate; 3, 4 — cured with pyrophosphate

Organoleptic indices were the best in samples with *PP* packed in vacuum. The quality of such meat was not altered up to 30 days without and up to 60 days after irradiation. Meat cured with *PP* and packed under atmospheric pressure without irradiation has kept up to 20 days while the irradiated one up to 60 days.

Samples of choice cooked sausage of the highest quality produced from meat cured with *PP* addition, non-irradiated and stored for 20 days or for 30—60 days when irradiated, obtained good scores on testing.

The results of the investigations permit us to assume that for the production of cooked sausage corned beef may be kept up to 20 days, at +4°C

without losses in technological properties, if packed in a polymer film in vacuum, while *PP* addition increases storage time up to 30 days.

Corned meat with and without *PP*, packed in vacuum and irradiated with 0.3 Mrad may be kept up to 60 days without losing its desirable properties.

2.3. Radurization of half-finished pork products

As seen from Fig. 8, peroxide number in irradiated pork is lower than the maximum tolerated by the standard over the entire storage period.

Accumulation of peroxide does not entirely reveal the real nature of the oxidative processes. It seemed to be interesting to follow the dynamics of

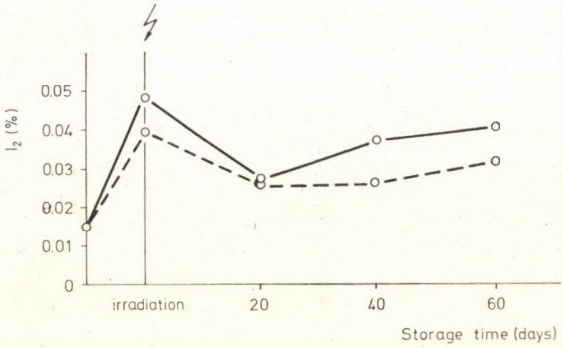

Fig. 8. Dynamics of changes in the peroxide number of irradiated pork during storage at 5°C. ——— untreated; - - - - - - treated with a mixture of NaCl, Na tripolyphosphate and ascorbic acid

accumulation of the secondary oxidation products, *i.e. CC*. The total amount of volatile *CC* in the initial meat constituted $149.0 \cdot 10^{-8}$ mole and increased four times by the end of storage (in untreated test samples).

Fifteen components were found in the volatile *CC* fraction by thin-layer chromatography. Glycol aldehyde, acetone, diacetyl, formaldehyde and acetaldehyde are present in prevailing quantities in the initial raw material. As for methylglyoxal, furfural and acetone they are present in lower amounts, other aldehydes such as propionic, butyric, valeric aldehydes are found in negligible quantities.

Under the influence of irradiation a marked, but differentiated increase in all *CC* occurs, which is especially significant in the case of diacetyl, formaldehyde, acetaldehyde, propionic, butyric, valeric, hexyl, heptyl, and caprylic aldehydes.

On further storage, and particularly during the period from the 20th to the 60th day all carbonyls are observed to increase.

This considerable increase in carbonyls in the period from the 20th to the 60th day may be explained by the activity of the viable microflora.

According to our preliminary data (ANISIMOV *et al.*, 1966) the amount of microorganisms in irradiated "schnitzel" reaches the value of 10^4—10^5 per g by the 20th day and increases up to 10^6—10^7 by the end of the storage period. As seen in Fig. 9, the *TBA* index sharply increases under the influence of irradiation and later on a gradual reduction in the malonic aldehyde level is observed. This can be explained, as pointed out by many authors, by its chemical binding due to its high reactivity.

Thus, during irradiation and storage of half-finished pork products a gradual accumulation of volatile *CC* takes place. However, their total amount does not, by the end of the 60th day, exceed (when expressed as carbonyl index) the value admissible for high-quality fat, even in test samples

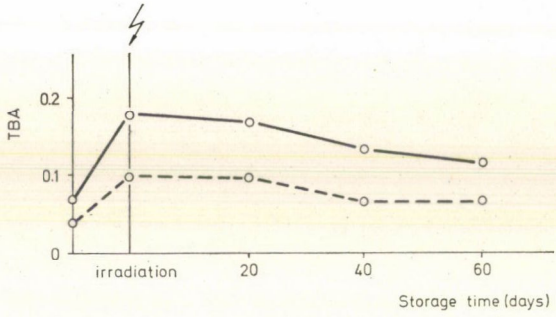

Fig. 9. Dynamics of changes in *TBA* during storage, at 5° C, of irradiated pork. ——— untreated; - - - - - - treated with a mixture of NaCl, Na tripolyphosphate and ascorbic acid

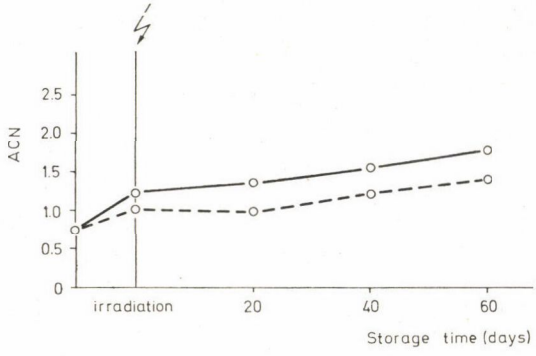

Fig. 10. Dynamics of the changes in acid number (*ACN* in mg KOH · g^{-1}) during storage of irradiated pork at 5°C. ——— untreated; - - - - - - treated with a mixture of NaCl, Na tripolyphosphate and ascorbic acid

with untreated surfaces. In samples treated with the mixture this index is even lower. Therefore, aldehydes and ketones formed during storage cannot affect the sensory properties of the product.

Alongside with *CC*, free fatty acids *(FFA)* are accumulated in the lipid fraction of meat, as a result of oxidative and hydrolytic processes, which may be characterized by the acid number *(ACN)*. A certain accumulation of *FFA* (Fig. 10) takes place during the process of irradiation which is explained fundamentally by lipase action. *ACN* does not exceed the tolerance limit of the standard.

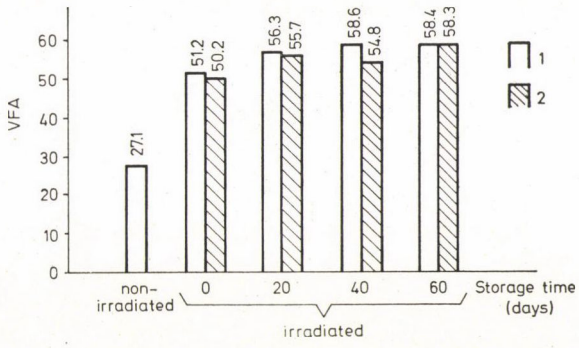

Fig. 11. Dynamics of the changes in volatile fatty acids (ml 0.01 *N* NaOH per 100 g dry product) during storage, at 5°C, of irradiated pork. 1 — untreated; 2 — treated with a mixture of NaCl, Na tripolyphosphate and ascorbic acid

Ionizing radiation promotes oxidative changes in fats which result in a considerable increase in *VFA* (Fig. 11).

Later on the accumulation of *VFA* is insignificant up to the end of storage. The differences in this index in the test and control samples are also negligible and this again proves that the initial increase in *VFA* is the result of irradiation but not of biochemical processes.

Eight components (Fig. 12) were found in the *VFA* fraction by means of gas-liquid chromatography.

Acetic acid was found in predominant quantities in the initial meat sample, while formic, propionic, isobutyric acid (giving a common peak), butyric and caproic acids are discovered in lesser quantities. Isovaleric and valeric acids are present in small quantities and caprylic acid only in traces.

The qualitative composition of *VFA* after irradiation and during the post-irradiation period remains constant, but some quantitative changes in the individual single components take place.

Butyric acid, isovaleric, valeric, caproic and caprylic acids are observed to increase considerably under the effect of irradiation (the first about four times).

Acetic acid, propionic and isobutyric acids change but slightly at any stage of the investigations of irradiated pork. Data obtained on the dynamics of *CC* and *VFA* accumulation widen our knowledge about the character of lipid changes due to irradiation of half-finished pork products with 0.6 Mrad and during further storage.

Chemical indices support the results of organoleptic investigations (METLITSKII *et al.*, 1967) and show that the changes in lipid components are not significant at low doses of irradiation and during the post-irradiation period.

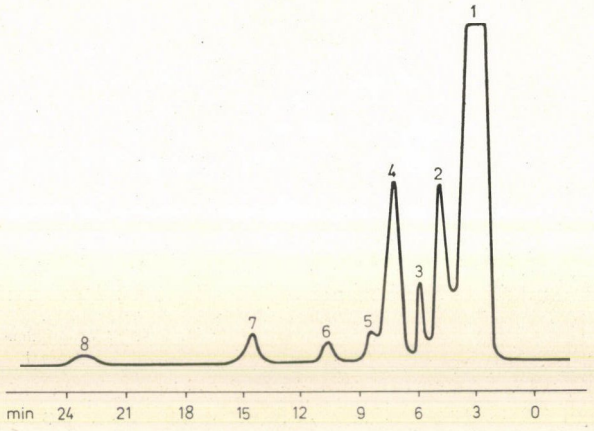

Fig. 12. Gas-liquid chromatogram of methyl esters of *VFA*. 1 — pentane; methyl esters of: 2 — acetic; 3 — propionic and isobutyric, 4 — butyric, 5 — isovaleric, 6 — valeric, 7 — caproic, 8 — caprylic acids

Irradiation combined with surface treatment of the product with the suggested mixture permits to prolong the storage life of half-finished pork products without reduction of their sensory qualities.

3. Conclusions

The experiments carried out have shown the possibility and expediency of using low doses of irradiation to facilitate the transport of chilled meat and to prolong the storage life of cured meat to be used for sausage production, and of low doses of irradiation to prolong shelf-life of half-finished pork products.

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IRRADIATION OF DATES

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Testing of the technical feasibility of radurization of fresh dates was attempted. In addition preliminary studies were carried out to investigate the applicability of gamma rays to date syrup manufacture.

The varieties *Zahdi, Lelwi* and *Tabarzel* were studied at different stages of ripening.

The eating quality of fresh dates was not affected significantly by irradiation even with doses of 270 and 540 krad.

The duration of the softening process (after-ripening) of dates was prolonged by low doses of 10—30 krad in the majority of the experimental batches. The time period of after-ripening was reduced with 270 krad, as well as with 540 krad as a result of shortening of the induction period (*i.e.* the time after which the date begins to soften).

The microbial spoilage of khalaal *Lelwi* dates was considerably reduced by irradiation with doses above 90 krad.

The dibis yield of fully rutab dates was highly increased by the radiation doses of 375 to 2000 krad. The darkness and viable cell count of dibis pressed from irradiated dates were significantly lower than that of untreated dates.

The facts that 37% of the date palms of the world is in Iraq and 75—80% of the international export of dates comes from Iraq (HUSSAIN, 1963; BARREVELD, 1971) underline the primary importance of date research in this country. Besides the eradication of insect infestation in dry dates (AHMED, 1970; AHMED *et al.*, 1970, 1971, 1972) the prolongation of the shelf-life of fresh dates would be economically very important (MAYAHI & AHMED, 1970). Fresh dates are preferred to dry dates by the local population but their distribution and availability are limited because of the perishability and short market life of these fresh fruits. Therefore, testing of technical feasibility of date radurization was attempted. Several experiments were carried out to study the applicability of gamma irradiation to date juice manufacture.

1. Materials and methods

1.1. Dates, their sorting, irradiation and storage

Three varieties of Iraqi dates were studied in three experimental series according to the experimental design shown in Table 1. The variety *Zahdi*

at different stages of ripening* is shown on Fig. 1. Fig. 2 shows the fruits of the *Lelwi* and *Tabarzel* varieties.

The freshly cut date bunches were purchased from the nearby Horticultural Experimental Station, Zafarania, and after their transport into the laboratory, the dates were picked. Next morning the dates were sorted according to their ripeness stage, then put into polyethylene pouches (max. 50 pieces per pouch) and irradiated at ambient temperature. A "Gammacell 220" unit was used as the radiation source. The dose rate changed from 468 to 463 krad per hour in the course of the experimental period.

After irradiation the dates were placed on polystyrene-foam trays and in palm-leaf baskets, resp. (Table 1) and stored at ambient temperature and

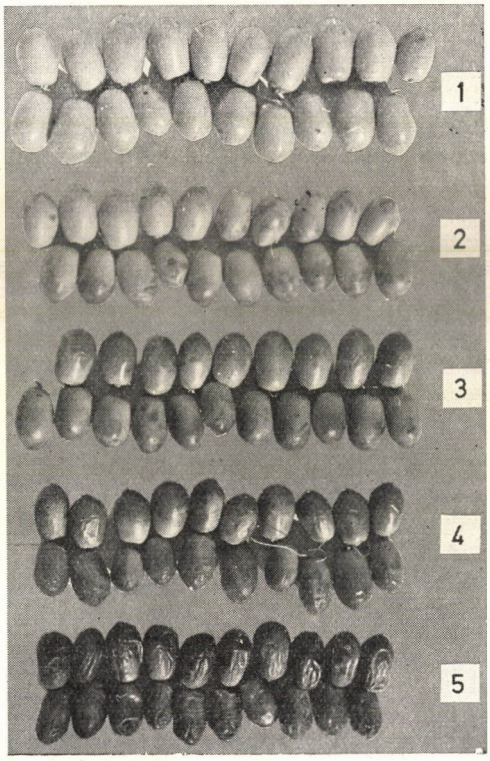

Fig. 1. The stages of after-ripening of *Zahdi* dates at the start of the first experiment. 1: Khalaal stage; 2: 10—30% rutab; 3: 50—70% rutab; 4: 70—90% rutab; 5: completely ripe

* Explanation of the ripeness-terms used:
khalaal: dates in fully grown, glossy, yellow stage, before they have become rutab
rutab: dates in their soft stage; this is the stage at which dates are sold for fresh consumption
tamar: dates in the fully-ripened, prune-like stage.

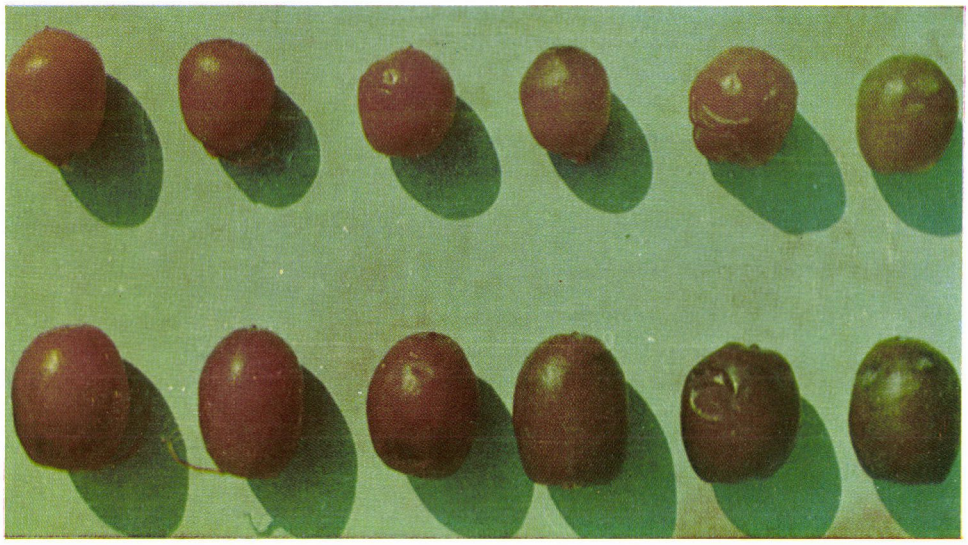

Fig. 2. Dates of the *Lelwi* (top row) and the *Tabarzel* (bottom row) varieties

Table 1

Design of the storage experiments with irradiated dates

Serial No. of experiments	Date of irradiation	Variety	Stage of ripeness at the time of irradiation	Radiation dose (krad)	Mode of storage	Aim of the study	Total number of fruits at each dose level
1	9. 10. 1972	*Zahdi*	khalaal	0; 10; 30; 90; 270	fruits on polystyrene-foam trays in separated holes	after-ripening, shelf-life, organoleptic quality	40
			10—30% rutab	0; 10; 30; 90; 270	,,	,,	40
			50—70% rutab	0; 10; 30; 90; 270	,,	organoleptic quality	40
			70—90% rutab	0; 10; 30; 90; 270	,,	,,	40
			completely ripe, dry	0; 10; 30; 90; 270	,,	,,	40
2	23. 10. 1972	*Tabarzel*	khalaal	0; 10; 30; 90; 270	fruits in palm-leaflet baskets (25 fruits in each)	after ripening, shelf-life, organoleptic quality	5 × 25
			0—20% rutab	0; 10; 30; 90; 270	fruits on polystyrene-foam trays in separated holes	after-ripening, shelf-life, organoleptic quality	50
3	4. 11. 1972	*Lelwi*	khalaal	0; 3.3; 10; 30; 90; 270; 540	fruits in palm-leaflet baskets (100 fruits in each)	after-ripening, shelf-life, organoleptic quality	4 × 100
			1—10% rutab	0; 3.3; 10; 30; 90; 270	fruits in palm-leaflet baskets (25 fruits in each)	after-ripening, shelf-life, organoleptic quality	25—75

humidity (between 16 and 29°C and 27—53% RH) on a shelf covered with a muslin net. This allowed ventilation but protected the samples from flies.

1.2. Determination of moisture content, equlibrium relative humidity and specific gravity of date-flesh

The moisture content of the edible portion of dates (sample size: 5.0 g of minced date-flesh) was estimated alternatively with a vacuum moisture tester with infrared heater (heating scale 4, for 30 min) and a distilling apparatus called Quickfit Water Estimator with light entrainer, using 50 ml toluene as the water-immiscible solvent. The duration of distillation was 60 min. It was shown in a preliminary experiment that the two alternative methods of moisture estimation give practically identical results (deviation less than 1% moisture).

The equilibrium relative humidity of date-flesh was estimated by the crystal liquefaction method (Vas & Proszt, 1955) at 25°C.

For estimation of specific gravity of the pitted date-halves a seven-grade series of sucrose solutions with known specific gravity was used (range from sp. gr. 1.03 to sp. gr. 1.29).

1.3. Sensory testing of dates

In organoleptic testing the triangular test as well as Kramer's ranking test were used. Significance of differences was evaluated according to Bengtsson (1953) and Kramer (1960), respectively.

1.4. Observations during storage of dates

During storage the samples were checked visually at intervals of several days and the percentage of ripeness of each fruit (the portion of the softened part of the fruit), as well as the number of shrivelled, spotted, moulded or fermented fruits were observed. The weight-loss of the samples was also determined.

1.5. Estimation of the length of after-ripening and the market life of fresh dates

From the results of observations described in para. 1.4 the average ripeness per cent of the samples was calculated and plotted against the storage time. Also, the percentage of the deteriorated dates in the samples was determined as a function of the storage time. For the quantal evaluation of the length of after-ripening and market life of fresh dates, probit transformation

(VAS, 1959, 1971) of ripening and spoilage curves, resp., was performed and from the corrected probit (WEBER, 1961) *vs.* storage time regression lines the time periods t_{16}, t_{50} and t_{84} (times in days elapsed between irradiation and 16, 50 and 84% ripeness and spoilage, respectively) were determined.

1.6. Estimation of softness of the date-flesh

Due to the lack of an instrument capable of testing textural characteristics, the following method was established to test the softness of the date-flesh.

The date was carefully peeled and its seed was removed with a forceps. A small sheet of a Whatman No. 41 filter paper was put on a wire mesh layed on the bench and the date-flesh was placed on the middle of the filter sheet and pressed with a weight of 1.0 kg for 20 seconds. Then the contours of the wet print were immediately marked. The print area of the date-flesh or preferably, the print area per unit weight of the date-flesh was found to be a satisfactory measure of the softness of the ripe fruit. The results were evaluated by analysis of variance.

1.7. Investigation of date syrup (dibis) release by pressure

To study the effect of irradiation on the yield of dibis (date syrup) *Tabarzel* and *Lelwi* dates in fully rutab stage were used. The dates were irradiated at different dose levels, peeled and pitted after irradiation, and 50 g amounts of the edible portion were put in porcelain Buchner-funnels. After covering them with a watch glass fitting in the inner size of the funnel the date-flesh was pressed with 1.5 kg weight for 24 hours at 27°C.

The dose levels for *Lelwi* dates in this dibis-release study were 0, 500, 1 000 and 2 000 krad, respectively. The radiation doses in a similar experiment with *Tabarzel* dates were 0, 375, 750 and 1 500 krad. In the case of *Lelwi* dates the pressure-test was started directly after irradiation. In order to study the reproducibility of the pressing method and the effect of post-irradiation storage, the pressure test was done not only directly after irradiation, but it was repeated with independent samples after 48 hours of storage following irradiation at room temperature. The amount of dibis released during the pressing procedure was determined, and the total soluble solids content measured with a hand-refractometer. The absorption spectra of dibis were recorded using Unicam SP 8000 spectrophotometer. In parallel with the dibis-pressing procedure, the tenderness of date-flesh was determined by the pressure-print method, described in para. 1.6.

2. Results

2.1. Organoleptic testing of dates

The *Zahdi* samples of the first date storage experiment (see Table 1) were scored two days after irradiation by 6 judges for juiciness and tenderness, respectively, according to the following score systems:

Juiciness:	extremely juicy	5 scores
	very juicy	4 scores
	juicy enough	3 scores
	not juicy enough	2 scores
	not juicy at all	1 score
Tenderness:	extremely soft	5 scores
	very soft	4 scores
	not soft, not hard	3 scores
	firm	2 scores
	too hard	1 score

The scores of each judge were transformed into rank numbers and the rank totals were calculated.

The average scores and rank totals are shown in Tables 2 and 3. As it can be seen from Table 3, no significant differences were found in juiciness and tenderness between irradiated and control dates in the same ripeness stage. No off-flavour was noticed by any member of the panel.

Table 2

Results of organoleptic testing of untreated and irradiated dates of the Zahdi variety, 2 days after irradiation

Ripening stage at the time of irradiation	Ripening stage at the time of sensory testing	Average scores											
		Juiciness						Firmness					
		0	10	30	90	270	Row average	0	10	30	90	270	Row average
		krad						krad					
khalaal	0.5—4.5% rutab	2.1	1.8	2.4	2.0	2.3	2.1	1.7	1.4	1.7	1.8	2.3	1.8
10—30% rutab	37—51% rutab	2.5	2.7	2.1	2.8	2.9	2.6	2.8	2.8	2.8	3.0	3.0	2.9
50—70% rutab	64—94% rutab	2.8	3.4	3.2	3.1	3.3	3.2	3.2	3.7	3.8	3.5	4.2	3.7
70—90% rutab	83—98% rutab	2.8	3.0	3.0	2.5	2.3	2.7	3.7	4.2	3.8	3.5	2.8	3.6
completely ripe, dry	completely ripe, dry	1.9	1.6	1.8	1.9	2.2	1.9	2.3	2.5	2.2	2.7	2.7	2.5

Table 3

Results of organoleptic testing of untreated and irradiated Zahdi dates, 2 days after irradiation

A. Juiciness

Ripening stage at the time of irradiation	Ripening stage at the time of organoleptic testing	Rank totals				
		0	10	30	90	270
		krad				
khalaal	0.5—4.5% rutab	18.0	22.5	13.5	20.5	15.5
10—30% rutab	37 —51% rutab	20.0	16.5	22.5	17.5	13.5
50—70% rutab	64 —94% rutab	23.5	16.0	16.0	20.0	16.0
70—90% rutab	83 —98% rutab	16.5	16.0	14.0	20.5	19.0
completely ripe, dry	completely ripe, dry	16.0	21.0	19.5	16.5	14.0

B. Firmness

Ripening stage at the time of irradiation	Ripening stage at the time of organoleptic testing	Rank totals				
		0	10	30	90	270
		krad				
khalaal	0.5—4.5% rutab	20.0	22.0	20.0	18.0	10.0
10—30% rutab	37 —51% rutab	18.0	22.5	17.5	16.0	16.0
50—70% rutab	64 —94% rutab	22.5	17.0	15.5	19.5	13.0
70—90% rutab	83 —98% rutab	14.0	14.5	17.5	18.5	25.5
completely ripe, dry	completely ripe, dry	18.0	15.5	22.0	15.5	15.0

Lowest insignificant rank sum: 10.0, highest insignificant rank sum: 26.0 ($\alpha \leq 0.05$)

The average score and rank sum for firmness of khalaal dates irradiated with 270 krad (underlined in Tables 2 and 3, resp.) was just on the borderline of significance showing that the threshold dose for softening khalaal *Zahdi* dates is around this dose level.

Table 2 shows furthermore that the panel felt the tamar dates as least juicy and the khalaal dates as the hardest ones. The dates of 64—94% rutab stage were scored as the most soft and juicy ones indicating that this stage of ripening is probably of the highest consumer quality.

In the second storage experiment (see Table 1) *Tabarzel* dates irradiated at the khalaal stage were tested the day after irradiation by a panel of 6 judges by the triangular method. The texture and flavour of irradiated samples at each dose level were compared with those of the untreated samples. No significant differences were found.

In the third storage experiment the general eating quality of *Lelwi* dates irradiated with 270 and 540 krad, respectively, was compared to that of the unirradiated dates by the triangular taste-testing method. The dates were irradiated in the khalaal stage and stored for 14 days before taste-testing. Therefore, all samples were in the 100% rutab stage at the time of organoleptic testing. Again, no significant difference was found between control and irradiated samples.

2.2. The effect of irradiation on the after-ripening of dates

It was shown by the first storage experiment that many of the *Zahdi* dates picked from their bunch in the khalaal stage became shrivelled, tough-dry during storage because they were unable to reach the fully rutab stage. The 270-krad dose somewhat stimulated the after-ripening of these immaturely picked fruits as shown in Fig. 3 illustrating the percentage distribution of the individual fruits according to their ripeness stage, after 8 and 16 days of storage, resp. Nevertheless, a considerable part of the 270-krad batch remained also in the immature, inedible stage.

When the *Zahdi* dates were picked from the bunch and irradiated in 10—30% rutab stage, after-ripening of both control and irradiated samples proceeded during storage. The speed of ripening of these samples was slowed

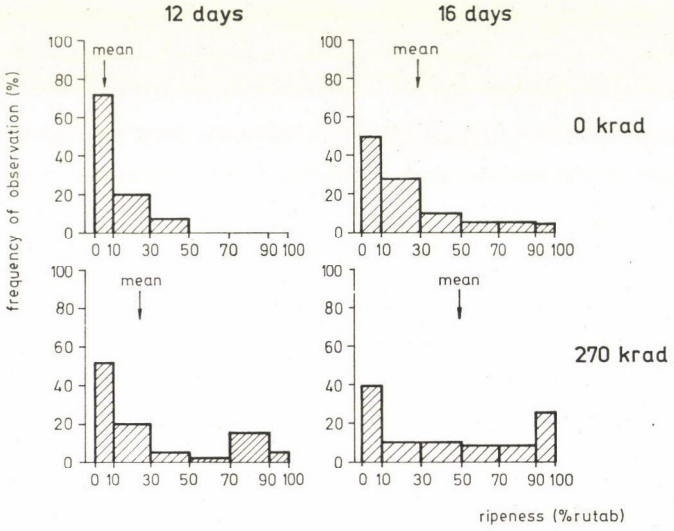

Fig. 3. Frequency distribution of ripeness in the experimental lots of *Zahdi* dates irradiated in the khalaal stage. Irradiation on 9 October 1972. Storage afterwards at 25—29°C and 27—45% RH. The distribution pattern was registered after 12 and 16 days of storage, resp.

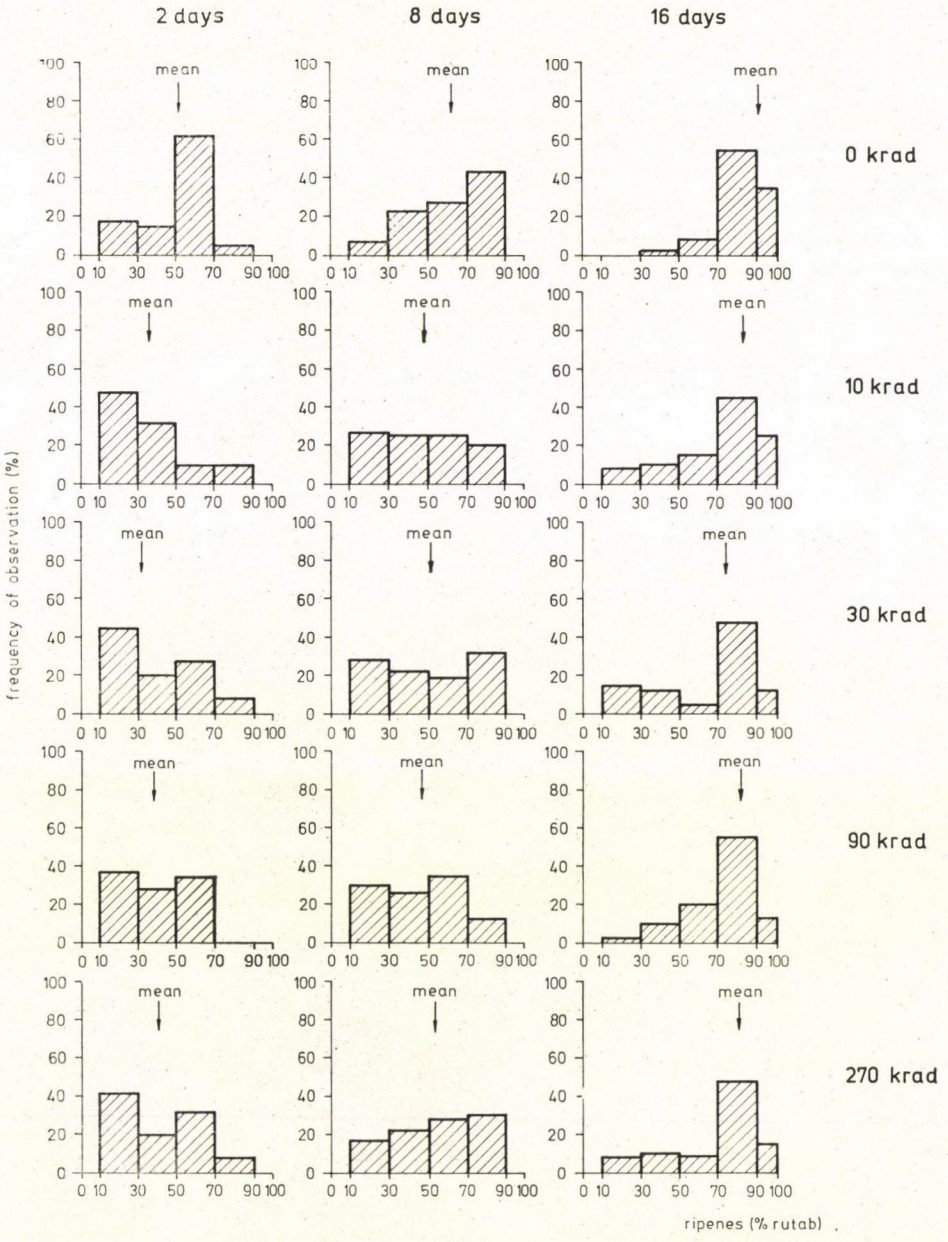

Fig. 4. Frequency distribution of ripeness in the experimental lots of *Zahdi* dates irradiated in 10—30% rutab stage. Irradiation on 9 October 1972. Storage afterwards at 25—29°C and 27—45% RH. The distribution pattern was registered after 2, 8 and 16 days of storage, resp.

down at each dose level as is shown in Fig. 4 and Fig. 5. Using the time-period elapsing between the 16 and 84% rutab stage as a measure of the duration of softening and of the highest consumer quality of fresh dates, it could be concluded from Fig. 5 that the optimum dose of 30 krad increased this time-interval in this particular case from about 13 days to 22 days.

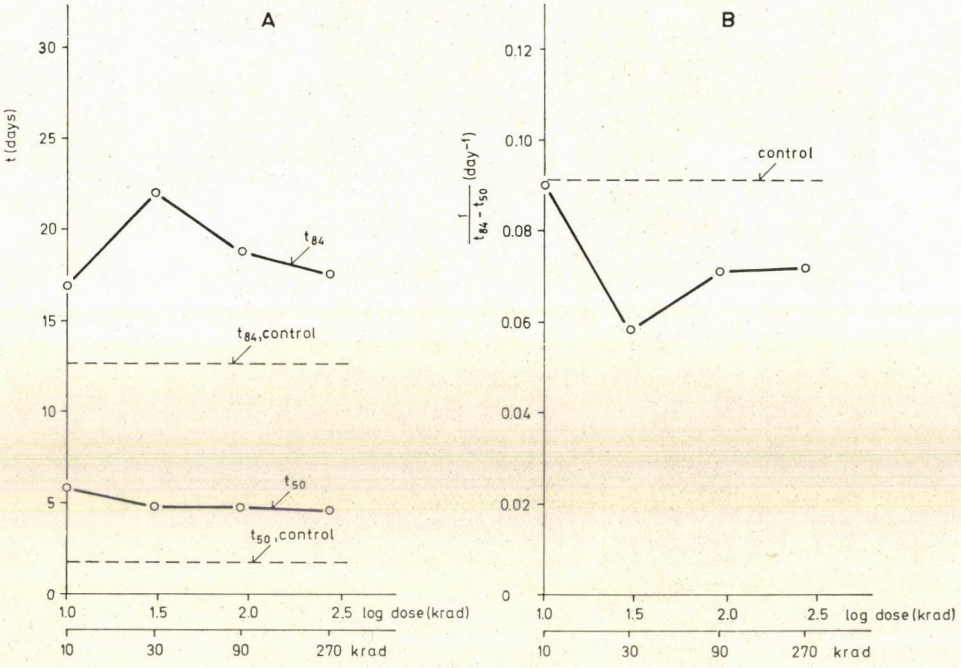

Fig. 5. *A*: Time elapsed between irradiation and the stage of 50 and 84% average ripeness of *Zahdi* dates, resp., as a function of the radiation dose. *B*: The speed of after-ripening of *Zahdi* dates $\left(\frac{1}{t_{84}-t_{50}}\right)$ as a function of the radiation dose. The fruits were irradiated on 9 October 1972 in 10—30% rutab stage and stored afterwards at 25—29°C and 27—45% RH

The *Tabarzel* dates investigated in the second storage experiment were able to reach complete after-ripening from the khalaal stage, too. As may be seen in Figs. 6, 7 and 8, the low dose treatments of 10—30 krad slightly reduced the speed of ripening while the 270-krad dose initiated softening without influencing the speed of ripening (the duration of the softening period). The t_{84} values of *Tabarzel* dates irradiated in 0—20% rutab stage are illustrated in Fig. 9. As it can be seen, here again 30 krad decreased the speed of after-ripening, while 90 and 270 krad increased it. Representative samples taken both from the untreated and the 270-krad irradiated lots are shown in Fig. 10 after 8 days of storage.

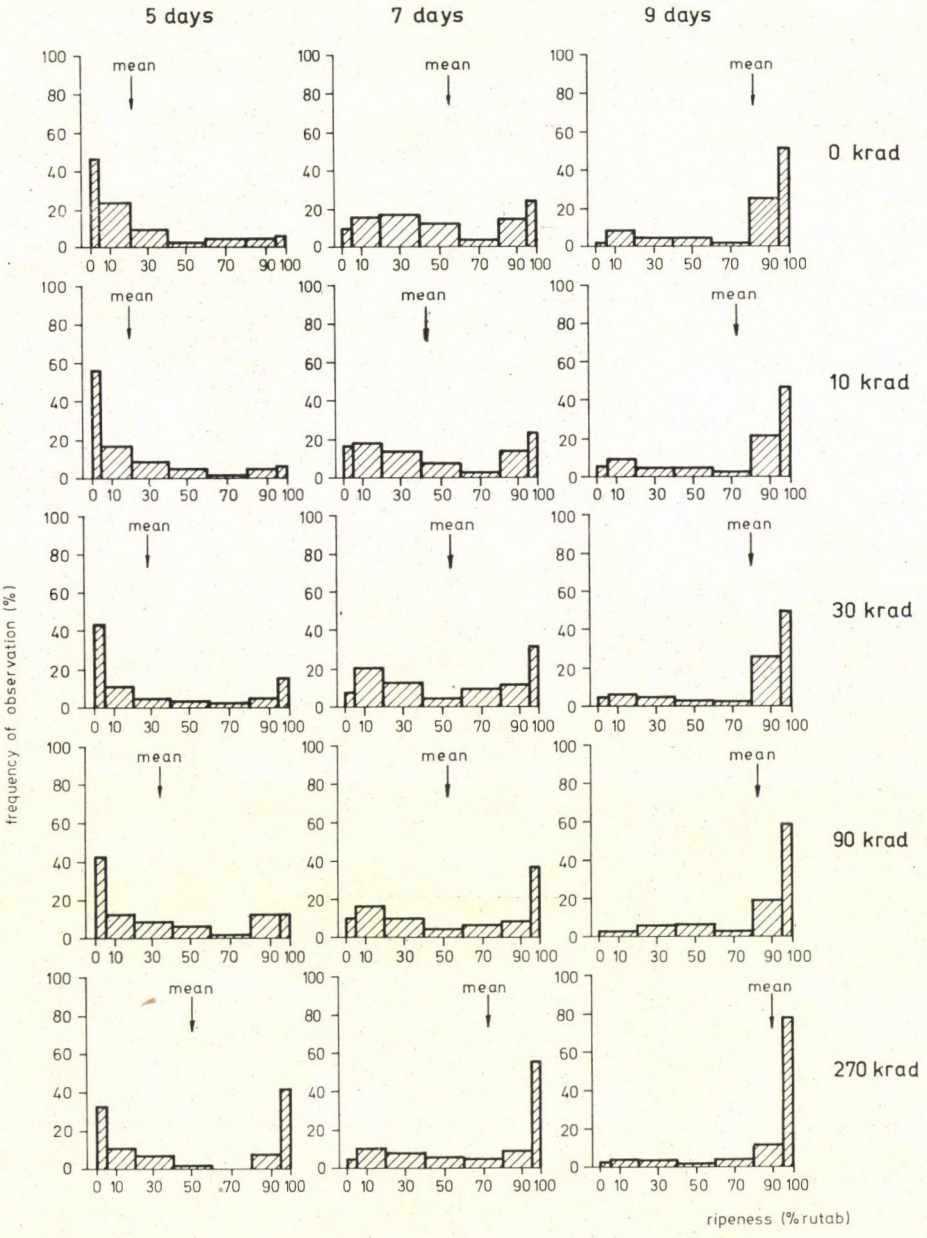

Fig. 6. The frequency distribution of ripeness in the experimental lots of *Tabarzel* dates irradiated in the khalaal stage. Irradiation on 23 October 1972. Storage afterwards at 22—28°C and 28—43% RH. The distribution pattern registered after 5, 7 and 9 days of storage, resp.

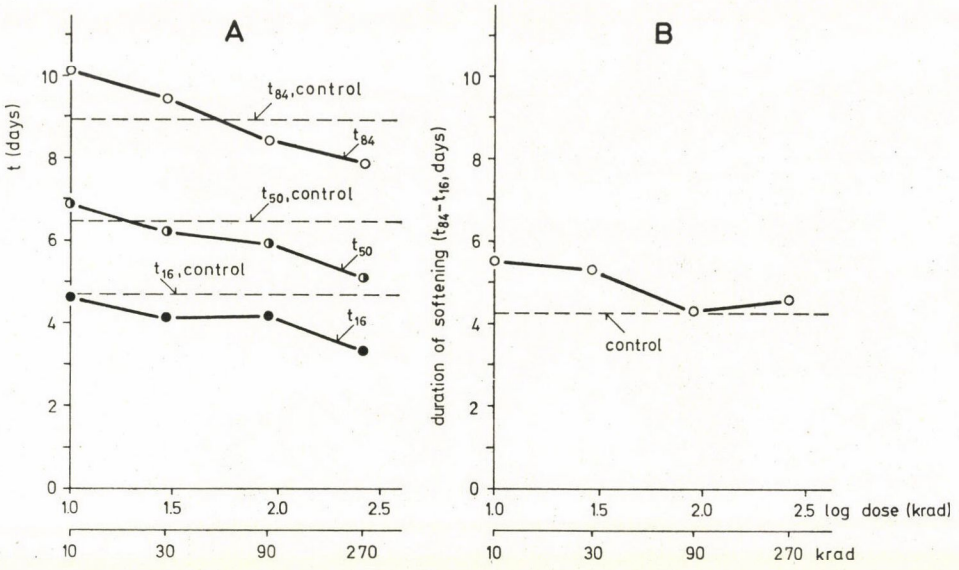

Fig. 8. *A*: Time elapsed between irradiation and the stages of 16, 50 and 84% average ripeness, resp., of *Tabarzel* dates, as a function of the radiation dose, *B*: The duration of softening (time between the stages of 16% and 84% average ripeness) of *Tabarzel* dates as a function of the radiation dose. The fruits were irradiated on 23 October 1972 in the khalaal stage and stored afterwards at 22—28°C and 28—43% RH

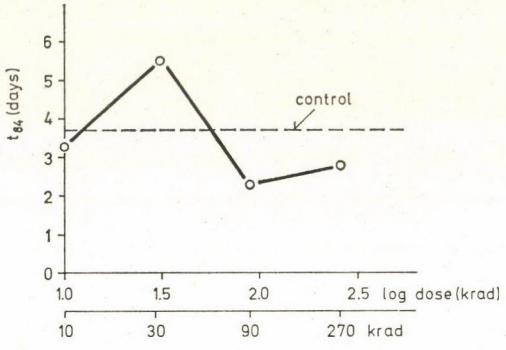

Fig. 9. Time elapsed between irradiation and stage of 84% average ripeness, of *Tabarzel* dates as a function of the radiation dose. The fruits were irradiated on 23 October 1972 in 0—20% rutab stage and stored afterwards at 22—28°C and 28—43% RH

The post-harvest softening of khalaal dates from the *Lelwi* variety studied in the third storage experiment was also stimulated by the increased radiation doses as shown in Fig. 11 and Fig. 12. In this experiment the tenderness of the dates was determined after 26 days of storage by measuring the print area of fruit flesh (see para. 1.6). The results are shown in Fig. 13 and Table 4. It turned out that the 270-krad sample was significantly softer than

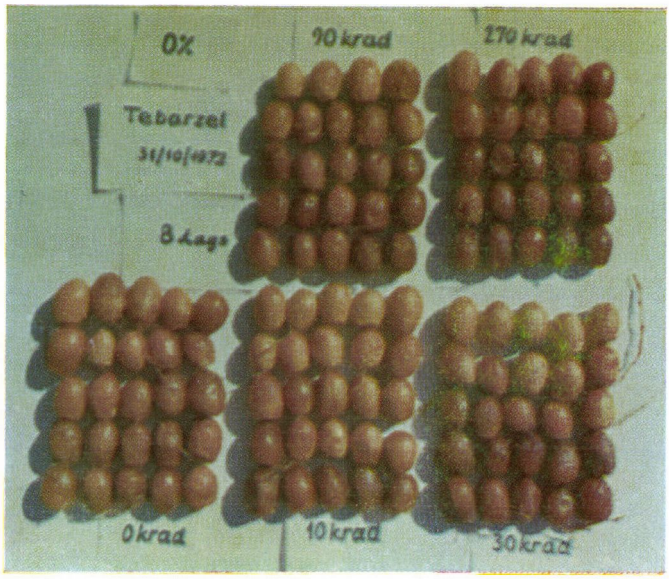

Fig. 7. The effect of irradiation on the after-ripening of *Tabarzel* dates. The samples were irradiated on 23 October 1972 in the khalaal stage. Photograph was taken after 8 days of storage at 22—28°C and 28—43% RH

Fig. 10. The effect of 270 krad on the after-ripening of *Tabarzel* dates, irradiated on 23 October 1972 in 0—20% rutab stage. The fruits were stored after irradiation at 22—28°C and 28—43% RH. The photograph was taken after 8 days of storage

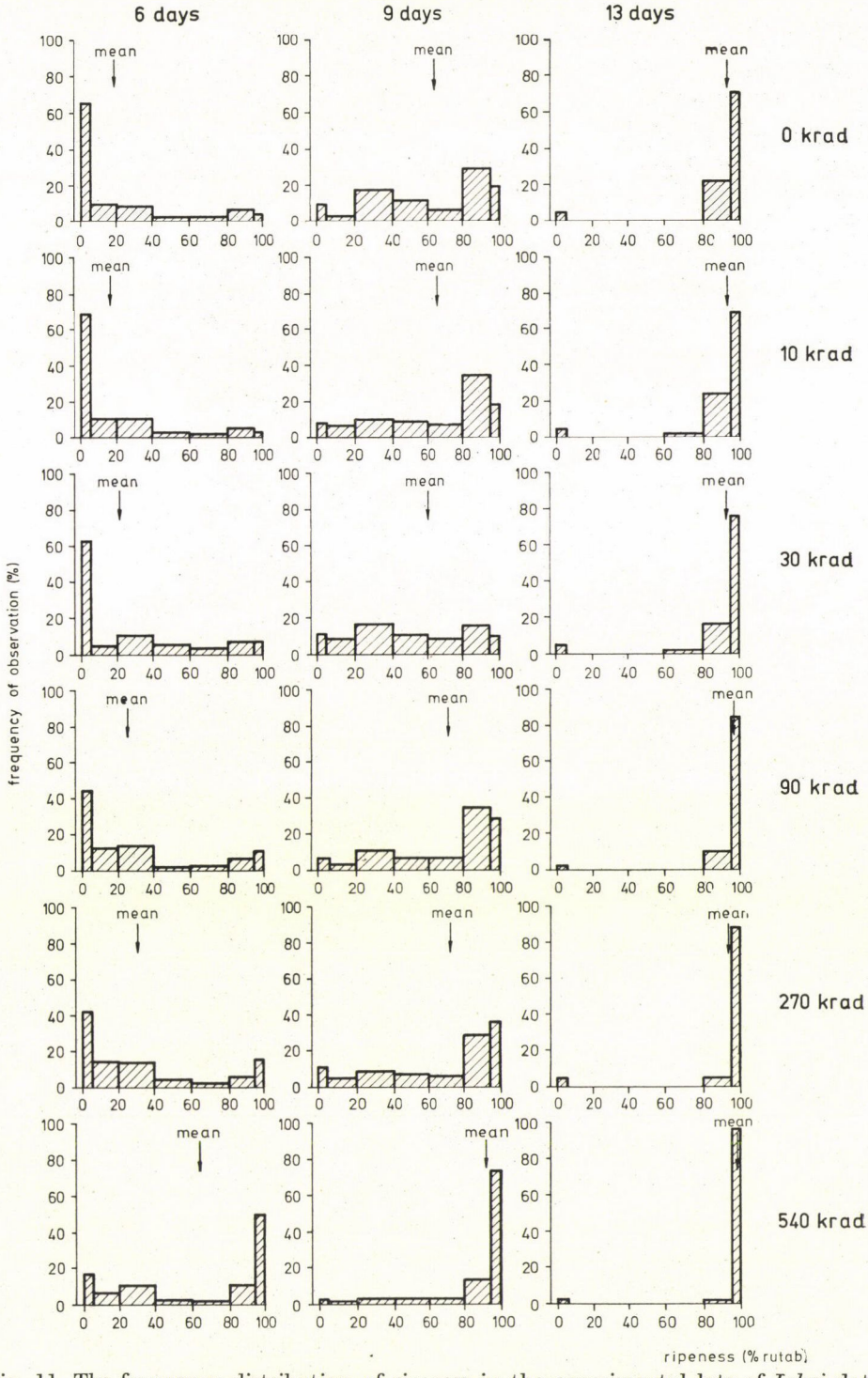

Fig. 11. The frequency distribution of ripeness in the experimental lots of *Lelwi* dates irradiated in the khalaal stage. Irradiation on 4 November 1972. Storage afterwards at 16—26°C and 33—53% RH. The distribution pattern was recorded after 6, 9 and 13 days of storage, resp.

the 3.3- and 10-krad samples. The 540-krad sample was significantly softer than the samples irradiated with 3.3 to 90 krad. The tenderness of the unirradiated sample was in between that of the low-dose treated samples and the high-dose treated ones. These results are in good agreement with the visual observation of the after-ripening illustrated in Figs. 11 and 12 and they strengthen the conclusion that the post-harvest softening of this variety

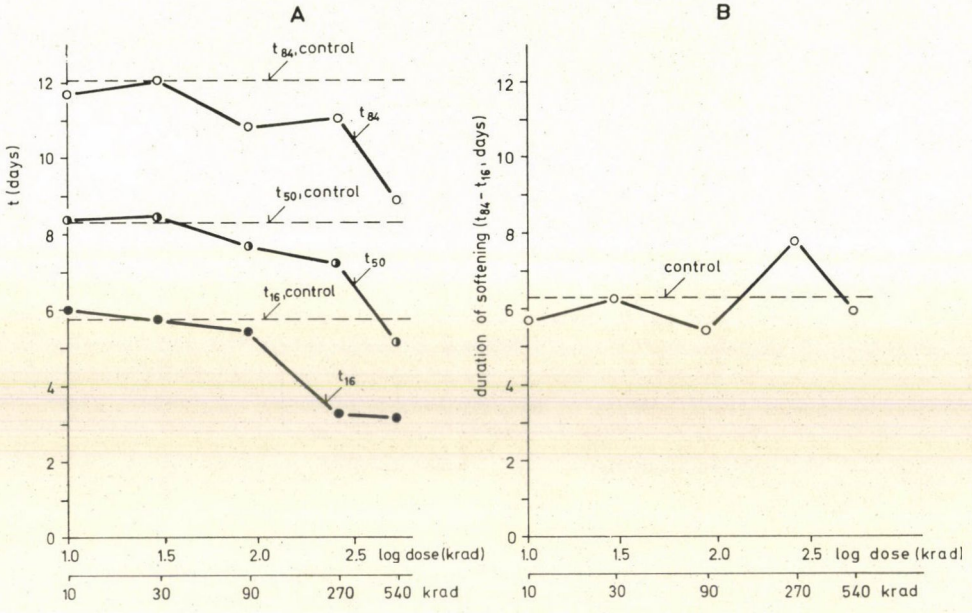

Fig. 12. *A*: Time elapsed between irradiation and the stages of 16, 50 and 84% average ripeness, resp., of *Lelwi* dates, as a function of the radiation dose; *B*: The duration of softening (time between the stages of 16 and 84% average ripeness) of *Lelwi* dates as a function of the radiation dose. The fruits were irradiated on 4 November 1972 in khalaal stage and stored afterwards at 16—26°C and 33—53% RH

irradiated in the khalaal stage was slightly inhibited by low-dose treatment and was promoted with doses of 270 and 540 krad. The storage experiment with *Lelwi* dates irradiated in 1—10% rutab stage showed again that the speed of after-ripening is reduced by 3.3 to 30 krad, while the 270-krad dose slightly stimulated the softening and browning processes (Fig. 14).

The very heterogeneous distribution of ripeness, among the dates picked in the khalaal stage (see Figs. 3, 6 and 11), indicates that sorting based on the surface colour was not satisfactorily selective (NADA, 1955). In future experiments, probably the specific gravity of dates could give a more selective estimate of the developmental stage. The estimation of specific gravity can

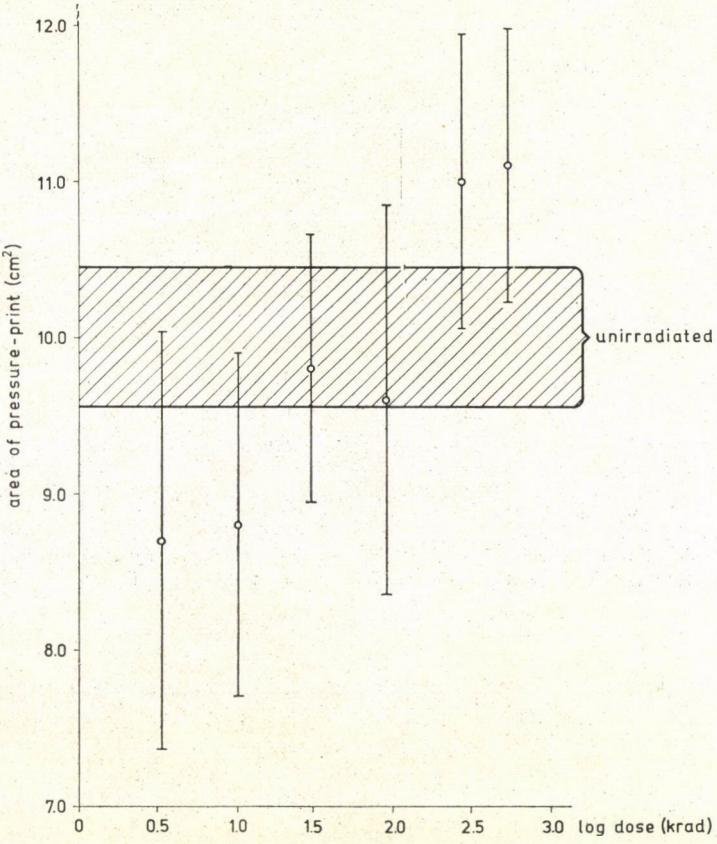

Fig. 13. The tenderness of the edible part of *Lelwi* dates after 26 days of storage as a function of the radiation dose. The tenderness is expressed as a pressure-print area, determined according to para. 1.6. The dates were irradiated in the khalaal stage on 4 November 1972 and stored afterwards at 26—26°C and 33—53% RH. The sample average plus-minus standard deviation is shown by the length of the bars

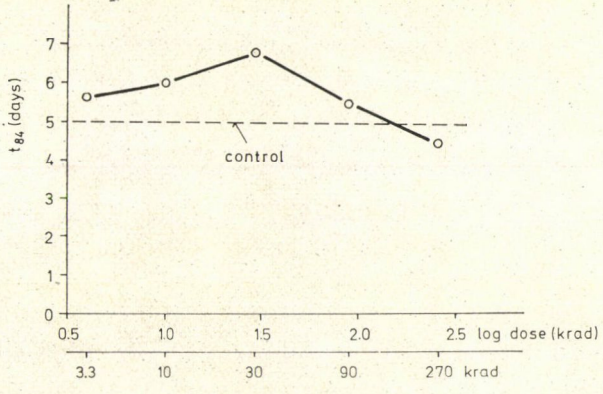

Fig. 14. Time elapsed between irradiation and the stage of 84% average ripeness of *Lelwi* dates, as a function of the radiation dose. The fruits were irradiated on 4 November 1972 in 1—10% khalaal stage and stored afterwards at 16—26°C and 33—53% RH

Table 4

*Tenderness of the edible part of Lelwi dates after 26 days of storage**

Radiation dose (krad)	Average print area of date-flesh (cm^2) and its standard deviation**
0	10.0 ± 0.4 abc
3.3	8.7 ± 1.2 a
10	8.8 ± 1.0 a
30	9.8 ± 0.8 a
90	9.6 ± 1.1 a
270	11.0 ± 0.8 bc
540	11.1 ± 0.8 c

* The dates were irradiated in the khalaal stage and they were in the fully rutab stage at the time of this investigation (see Fig. 9)

** Details of the investigation are described in para. 1.6. Any two means not followed by the same letter are significantly different from each other at the 5 per cent level

be performed quickly and simply by using a series of salt or sugar solutions to test the floating behaviour of the fruits.

2.3. The effect of irradiation on the deterioration of fresh dates

In the first storage experiment no microbial spoilage occurred in the dates during the 20-day period of the storage study. The percentage of shrivelled *Zahdi* dates in the samples picked and irradiated in the khalaal stage increased during storage. Fig. 15 shows that early shrivelling was reduced by irradiation. None of the *Zahdi* dates picked and irradiated in the 10—30% rutab stage were shrunk and all of them were edible after 20 days of storage.

Unlike in the case of the semi-dry type *Zahdi* variety, no tough-dry shrinkage occurred in the khalaal picked fruits of the soft varieties *Tabarzel* and *Lelwi*. The portion of moulded dates among the *Tabarzel* samples was less than 2 per cent in both irradiated and control batches after 12 days of storage.

In the third storage experiment, when 100 fruits per basket were stored of the khalaal *Lelwi* dates, microbial spoilage (moulding or fermentation) occurred from the second week of storage and became the predominant phenomenon of deterioration. The time periods elapsed for reaching levels of 5, 16 and 50 per cent deterioration of the samples (t_5, t_{16} and t_{50} values) are illustrated in Fig. 16 as a function of the radiation dose. From the point of

view of commercial practice, the 5% spoilage level could serve as a measure of the shelf-life of dates. Taking this value into consideration, the shelf-life of these samples was considerably increased by doses of 90 krad and above.

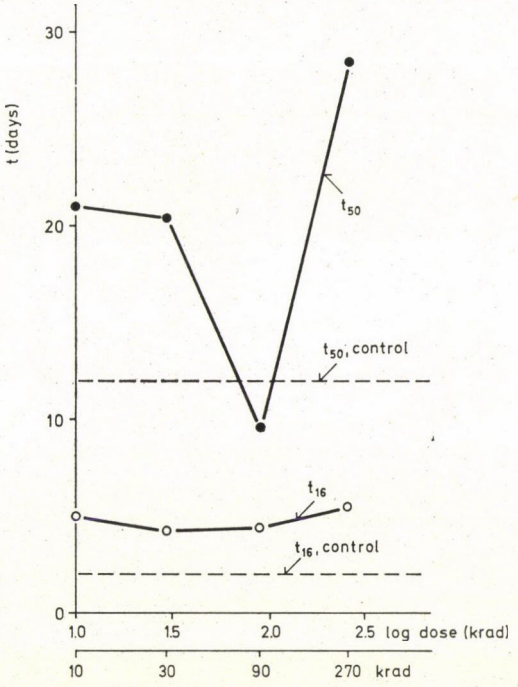

Fig. 15. Time elapsed between irradiation and the stages of 16 and 50% shrivelling of the experimental lots of khalaal *Zahdi* dates as a function of the radiation dose. The fruits were irradiated on 9 October 1972 and stored afterwards at 25—29°C and 27—45% RH

Table 5

Spoilage of Lelwi dates picked and irradiated in the khalaal stage

Radiation dose (krad)	Spoilage per cent at the time when spoilage in the control samples reached the 5 per cent level
0	5.0
3.3	3.9
10	4.8
30	4.1
90	2.7
270	2.2
540	1.0

As can be seen in Table 5, spoilage was as low as 1.0 per cent in the 540-krad samples, and 2.2% in the 270-krad samples at the t_5-time value of the control samples. Until this time the dates showed a weight loss of about 11 per cent of their original weight, independently of the radiation dose.

The *Lelwi* dates picked and irradiated when the softening has already started, remained almost 100% edible, without any visible microbial deterioration in the 50-day period of observation.

Table 6

The effect of irradiation on after-

Variety	Ripening stage at the time of irradiation	Storage conditions		Radiation doses (krad)	After-ripening (days)				
		temper. °C	rel. humid. %		t_{16}	t_{50}	t_{84}	$t_{84}-t_{16}$	$t_{84}-t_{50}$
Zahdi	khalaal	25—29	27—45	0 10 30 90 270	not evaluated because of shrivelling of the dates				
	10—30% rutab	25—29	27—45	0		1.8	12.7		10.9
				10		5.9	16.9		11.0
				30		4.8	22.0		17.2
				90		4.8	18.8		14.0
				270		4.6	17.6		13.0
Tabarzel	khalaal	22—28	28—43	0	4.7	6.5	9.0	4.3	
				10	4.7	6.9	10.1	5.4	
				30	4.1	6.2	9.5	5.4	
				90	4.2	5.9	8.4	4.2	
				270	3.3	5.1	7.9	4.6	
	0—20% rutab	22—28	28—43	0			3.7		
				10			3.3		
				30			5.5		
				90			2.3		
				270			2.8		
Lelwi	khalaal	16—26	33—53	0	5.8	8.3	12.1	6.3	
				3.3	not observed				
				10	6.0	8.4	11.7	5.7	
				30	5.8	8.4	12.1	6.2	
				90	5.4	7.7	10.9	5.4	
				270	3.3	7.2	11.1	7.8	
				540	3.2	5.2	8.1	5.9	
	1—10% rutab	16—26	33—53	0			5.0		
				3.3			5.6		
				10			6.0		
				30			6.8		
				90			5.5		
				270			4.5		

The findings concerning the effect of irradiation on the after-ripening and deterioration of the three date varieties are summarized in Table 6.

2.4. Weight loss of fresh dates during storage

Gamma irradiation with doses of 3.3 to 540 krad did not affect significantly the weight loss of dates during storage.

In the first storage experiment, it was observed that the *Zahdi* dates picked in the khalaal stage being unable to ripen to the fully rutab stage

ripening and deterioration of fresh dates

Deterioration time (days)					Conclusions
Shrivelling		Microbial spoilage			
t_{16}	t_{50}	t_5	t_{16}	t_{50}	
2.0 5.0 4.3 4.4 5.5	12.0 21.0 20.4 9.8 28.5	no spoilage in 30 days of storage			khalaal *Zahdi* dates are unable to after-ripen, early shrivelling is reduced, after-ripening is stimulated by irradiation
shrivelling did not occur		no spoilage in 30 days of storage			speed of after-ripening is reduced by irradiation, the optimum dose is 30 krad
shrivelling did not occur		no spoilage in 14 days of storage			speed of after-ripening is reduced by 10—30 krad; 270 krad can initiate the ripening process but does not decrease the speed of after-ripening
shrivelling did no toccur		no spoilage in 14 days of storage			speed of after-ripening is reduced by 30 krad. After-ripening is stimulated by doses of 90 and 270 krad
shrivelling did not occur		11.4 13.2 11.9 13.1 16.5 18.0 21.5	23.2 22.6 25.6 24.8 27.8 29.3 30.8	41.7 37.6 47.3 42.9 45.5 47.0 45.5	270 and 540 krad induce after-ripening. The speed of after-ripening is decreased by 270 krad. 90 krad and higher doses decreased the microbial spoilage of dates
shrivelling did not occur		no spoilage in 50 days of storage			speed of after-ripening is reduced by 3.3 to 30 krad. The 270 krad dose slightly stimulates after-ripening

showed a considerably higher weight loss than the dates picked in more progressed stages of ripening (Fig. 17). Similar tendency was observed in the second and third storage experiments, *i.e.* the riper the dates were picked, the lower was the velocity of weight-loss during storage.

Determination of moisture of *Zahdi* date flesh indicated that the moisture content was about 47% in the khalaal fruits, around 33% in the 50% rutab dates and ca. 30% when the fruits reached their fully rutab stage. The specific

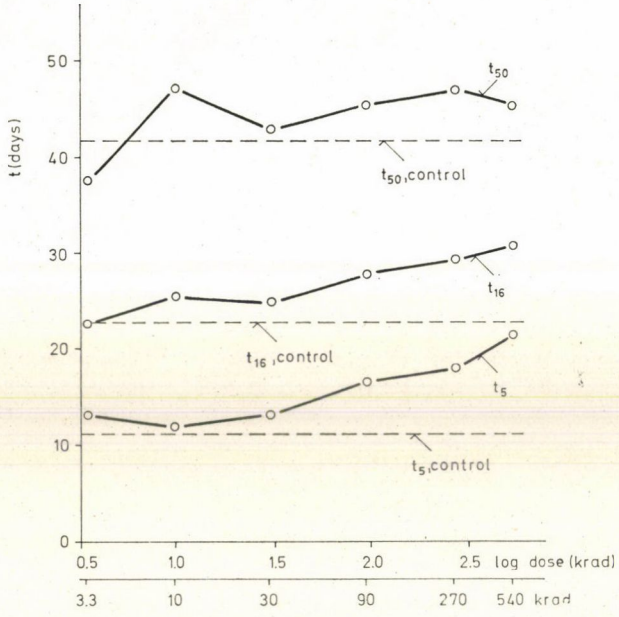

Fig. 16. Time elapsed between irradiation and the stages of 5, 16 and 50% spoilage of the experimental lots of *Lelwi* dates as a function of the radiation dose. The fruits were irradiated in the khalaal stage and stored afterwards at 16—26°C and 33—53% RH

gravity of the edible portion was 1.04—1.05 g/cm³ for the khalaal, 1.13—1.15 g/cm³ for the 50% rutab and 1.18—1.22 g/cm³ for the fully rutab *Zahdi* dates.

The specific gravity of the soft tip end of 50% rutab dates was nearly equal to that of fully rutab fruits while the unripened part (calyx end) showed practically the same specific gravity as the khalaal flesh. This fact points to an inhomogeneous moisture distribution in the dates during the transition period, well demonstrated by TURRELL and his co-workers (1940).

The moisture content varied around 37% in the fully rutab fruits of the *Lelwi* variety (sp. gr. 1.20—1.25) and about 35% of the *Tabarzel* variety.

The equilibrium relative humidity of the edible part of *Zahdi* dates was as follows:

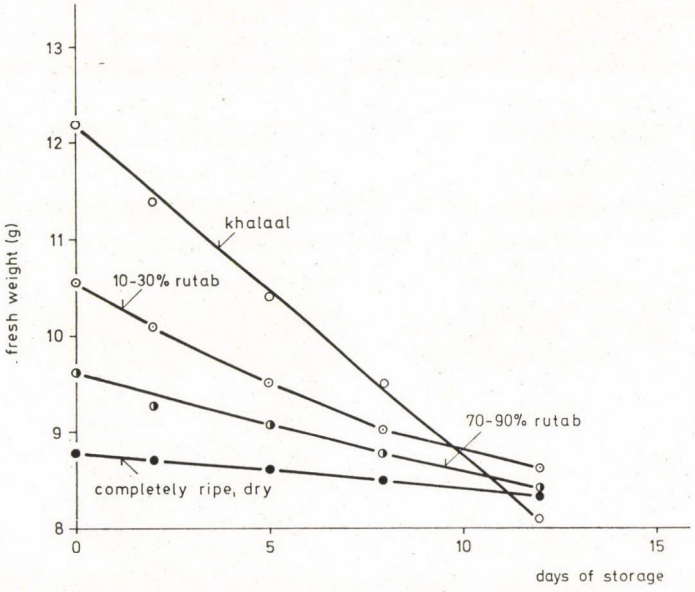

Fig. 17. Weight loss of *Zahdi* dates as a function of storage time and ripeness stage at the time of picking. The fruits were stored at 25—29°C and 27—47% RH on polystyrene-foam trays in separated holes

khalaal stage: 49.0% moisture and 92.8% ERH,
fully rutab stage: 30.4% moisture and 75.5% ERH.

These ERH data are in very good agreement with the ones published by RYGG (1948).

2.5. The applicability of gamma irradiation to date juice manufacture

2.5.1. The yield of dibis from fully rutab dates as affected by gamma irradiation. The dibis yields of *Lelwi* and *Tabarzel* dates in the pressing procedure described in para. 1.7 are shown in Table 7 and Table 8, respectively.

In both cases, the dibis release was drastically increased by irradiation. It is interesting to note that the highest yield of dibis was obtained not at the highest dose levels of 1 500 to 2 000 krad but with the medium doses of 750 to 1 000 krad. To explain this phenomenon, more exhaustive observations are needed. The statistical analysis of the results with *Tabarzel* dates showed that the dibis release was not significantly affected by the 48-hour post-irradiation storage period.

2.5.2. Colour and sugar-concentration of dibis. Irradiation bleached the brown colour of the fully rutab date flesh and the colour of dibis released from irradiated dates was lighter than that of the control.

Table 7

Dibis yield of fully rutab Lelwi dates as affected by gamma irradiation

Radiation dose (krad)	Dibis yield of 50 g date flesh of 37% moisture content, pressed with 1.5 kg weight for 24 hours at 27°C	
	g	%
0	7.10	100.0
500	9.40	132.3
1 000	10.15	143.0
2 000	9.05	127.5

Table 8

Dibis yield of fully rutab Tabarzel dates as affected by irradiation

The period of postirradiation storage of dates (hours)	Dibis yield of 50 g date-flesh of 35% moisture content, pressed with 1.5 kg weight for 24 hours at 27°C (g)			
	0 krad	375 krad	750 krad	1 500 krad
0	6.60	9.80	11.00	8.45
48	5.40	9.50	11.30	9.60
Mean value ± standard deviation	6.00±0.85 a	9.65±0.22 b	11.15±0.22 c	9.03±0.81 b
Mean values as a percentage of the control	100.0	160.8	185.8	150.6

Any two means not followed by the same letter are significantly different from each other ($\alpha \leq 0.05$)

The total soluble solids content of dibis measured with a refractometer showed a random variability, but the concentration range for the two varieties was practically the same, between 62.5 and 65%.

The absorbance values (A) of undiluted dibis samples measured in a UNICAM SP 8000 spectrophotometer (path length 10 mm) are shown in Table 9 and Table 10. Since absorbance values of the undiluted control samples under a wavelength of 530 nm were higher than 1.0 A (the upper limit of the measuring range), the differential spectra between dibis samples obtained from control and irradiated *Tabarzel* dates were determined, using the dibis from irradiated sample as reference. These differential spectra showed an absorption maximum at about 440 nm indicating that the brown pigment content of the dates was diminished by irradiation.

Table 9

Optical density of dibis released from Lelwi dates irradiated in the fully rutab stage

Radiation dose (krad)	Total soluble solids (%)	Absorbance at 540 nm ($A_{540\ nm}$)
0	62.5	0.960
500	64.1	0.595
1 000	62.8	0.675
2 000	63.4	0.610

Table 10

Optical density of dibis released from Tabarzel dates irradiated in fully rutab stage

Radiation dose (krad)	Total soluble solids (%)	$A_{540\ nm}$	$A_{750\ nm}$	$A_{control}-A_{irrad.}$ at 440 nm
0	65.0	0.823	0.134	—
375	63.1	0.620	0.154	0.340
750	64.0	0.610	0.113	0.315
1 500	62.7	0.508	0.153	0.485

2.5.3. Sensory quality of the dibis samples. The undiluted dibis samples released from control and irradiated dates, resp., were scored by taste testing for organoleptic quality with 6 judges, according to the following hedonic scale:

5 like extremely
4 like
3 neither like nor dislike
2 dislike
1 dislike extremely.

The scores were evaluated by Kramer's ranking method (KRAMER, 1960). The results are shown in Table 11. It can be seen that all samples were of a good quality and no significant differences were found between dibis samples pressed from control and irradiated dates.

2.5.4. Microbiological quality of dibis samples released from irradiated and control Lelwi dates, respectively. The results of the surface plating of dibis samples are shown in Table 12. In spite of the fact that the dibis samples were produced and stored under non-aseptic conditions, the viable counts of samples from

irradiated dates were about two orders of magnitude lower than that of a dibis sample produced from unirradiated dates. The mould infection of dibis consisted predominantly of *Aspergillus niger*. The non-mould colonies consisted mainly of yeasts and cocci.

2.5.5. Tenderness of dates irradiated in the fully rutab stage. The specific print area values obtained by the pressure-print method described in para. 1.6 were evaluated by analysis of variance. This evaluation showed that the tenderness of date-flesh was significantly affected by the radiation treatment. It can be seen in Fig. 18 that the softness of the date-flesh was increased with increasing doses. The degree of change was greater in the less soft *Tabarzel* dates than in the *Lelwi* dates which had been very soft already before irradiation. These results, pointing to the degradation of structural polysaccharides of the dates by irradiation, are in good agreement with the results of the increased dibis yield of irradiated dates.

Table 11

Taste testing of dibis samples released from unirradiated and irradiated dates, respectively

	Tabarzel dates				*Lelwi* dates			
	0	375	750	1 000	0	500	1 000	2 000
	krad				krad			
Average score	4.33	3.77	4.00	4.50	3.33	4.17	4.67	4.50
Rank total	16.0	21.0	18.5	15.5	21.0	18.0	15.5	15.5

Rank totals required for significance at the 5% level:
lowest insignificant rank sum: 9
highest insignificant rank sum: 21

Table 12

Colony counts of dibis samples from irradiated and control Lelwi dates, resp.

Radiation dose (krad)	Colony count/ml dibis					
	on Bacto-Nutrient agar		on Sabouraud agar		on Wickerham agar	
	mould count	non-mould count	mould count	non-mould count	mould count	non-mould count
0	2.7×10^2	$>10^5$	2.7×10^2	$>10^5$	2.5×10^2	$>10^5$
500	3.0×10^0	4.8×10^2	$< 3.0 \times 10^0$	1.2×10^3	7.0×10^0	1.4×10^3
1 000	$< 3.0 \times 10^0$	1.9×10^2	$< 3.0 \times 10^0$	2.3×10^2	7.0×10^0	2.4×10^2
2 000	1.3×10^1	2.3×10^2	7.0×10^0	2.8×10^2	3.0×10^0	4.7×10^2

2.5.6. Radiation preservation of dibis juice. With freshly pressed dibis from fully rutab *Tabarzel* dates a small exploratory experiment was carried out to study the possibility of radurization of date juice.

In order to get faster spoilage, the dibis was diluted with tap water to 17.5% soluble solids (fourfold dilution of dibis), then distributed in 2-ml portions into small stoppered glass vials and equal numbers of vials were irra-

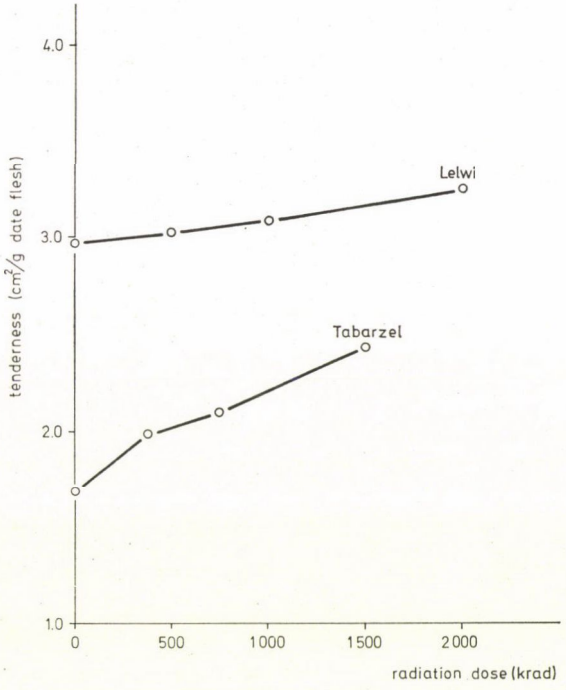

Fig. 18. Tenderness of the edible part of fully rutab *Tabarzel* dates and *Lelwi* dates, resp., as a function of radiation dose. The tenderness is expressed as the specific pressure-print area, measured according to para. 1.6. The moisture content of the date-flesh: 37% in *Lelwi* dates and 35% in *Tabarzel* dates

diated with 0, 375 and 750 krad, respectively. The pH of the samples was 5.9. Directly after irradiation, the viable cell count of one sample from each dose level was estimated by surface plating on Wickerham agar and Sabouraud agar media, respectively. With several other samples a taste testing procedure identical with the method described in para. 2.5.3 was performed. The remaining samples were incubated at ambient temperature to record spoilage.

The results of taste testing are shown in Table 13. It can be seen that the irradiated samples were of good organoleptic quality, no off-flavour was reported and no significant differences were found between the samples.

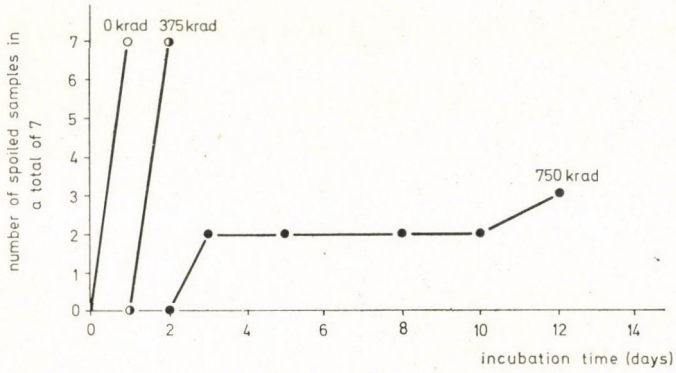

Fig. 19. The spoilage of diluted dibis samples as a function of the radiation dose and incubation time at ambient temperature. The dibis was pressed from fully rutab *Tabarzel* dates of 35% moisture content and diluted fourfold before radiation treatment

Table 13

Taste testing of date juices of 17.5% soluble solids content, directly after irradiation
Number of judges: 6

	0	375	750
	krad		
Average score*	4.33	3.60	4.00
Rank total**	9.0	14.5	12.5

* The hedonic scale of scores is described in para. 2.5.3.
** Rank totals required for significance at the 5% level:
lowest significant rank sum: 8
highest significant rank sum: 16

The colony counts after 96 hours of incubation at 27°C are shown in Table 14. The microscopic observation of isolates proved that the dominant survivors were yeasts in the 375-krad samples, and cocci in the 750-krad samples.

Table 14

Colony counts of date juices of 17.5% solids content directly after irradiation

Radiation dose (krad)	Colony count/ml juice			
	on Wickerham agar plates		on Sabouraud agar plates	
	mould	non-mould	mould	non-mould
0	1.8×10^3	2.0×10^4	2.0×10^3	1.5×10^4
375	$< 1.0 \times 10^1$	8.5×10^2	$< 1.0 \times 10^1$	9.0×10^2
750	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	1.0×10^1

The spoilage of samples was followed by recording the symptoms: turbidity, gas development, sediment and/or film formation. The ratio of spoiled samples as a function of the incubation time is shown in Fig. 19.

3. Conclusions

The results of the sensory tests (para. 2.1, Tables 2—3) show that the eating quality of dates is not affected significantly by irradiation, even with the doses of 270 and 540 krad. The same favourable results were observed with the dibis samples produced from dates irradiated up to 2 000 krad (para. 2.5.3, Table 11), as well as with the date juice (para. 2.5.6, Table 13) irradiated up to a dose of 750-krad. This insensitivity of dates and date products towards irradiation is probably due to the relatively low water activity and low protein content (DOWSON & ATEN, 1962; HASEGAWA *et al.*, 1969) of these products as compared with many other fruits and vegetables where the threshold for undesirable organoleptic changes was found in the range of a few hundred krad. Therefore, the organoleptic quality does not seem to be a limiting factor in the application of irradiation to date processing.

The studies on after-ripening of irradiated and control dates, resp., revealed considerable differences in the response of different varieties and developmental stages of the dates (see para. 2.2, Figs. 5—14 and Tables 4 and 6). This phenomenon is not uncommon with other fruits, too, and well documented in the food irradiation literature. For the sake of generalization it can be stated that the duration of the softening process was increased by low doses (10—30 krad) in the majority of the experimental batches (the speed of the after-ripening process was reduced). About 75% increase in softening time was observed with the 10—30% rutab *Zahdi* dates as well as with 1—20% rutab *Tabarzel* dates, about 25% increase was found with the khalaal *Tabarzel*, and 1—10% rutab *Lelwi* samples, while no increase was shown with khalaal *Lelwi* samples. The majority of khalaal *Zahdi* fruits were unable to after-ripen into the fully rutab stage, and shrivelling occurred. This phenomenon seems to be typical for the semi-dry varieties (RYGG, 1950).

The 270- as well as the 540-krad doses reduced the time period elapsing between irradiation and the 84% rutab stage of fresh dates. This stimulatory effect does not seem to be exerted on the speed of the physiological softening process already commenced, but rather on the initiation of softening (*i.e.* by shortening the induction period before commencing the softening of the dates picked in the khalaal stage). The induction period between the khalaal and rutab stages was 30% shorter with the 270-krad irradiated khalaal dates of the *Tabarzel* variety and 45 per cent shorter with the ones of the *Lelwi* variety. An increase in the number of partly rutab fruits was observed among

the 270-krad irradiated *Zahdi* fruits picked in the khalaal stage, which otherwise are unable to undergo complete after-ripening.

The experiences mentioned above are probably based on the differences of dose requirements for certain physiological, structural and chemical changes in the date fruit. The inhibition of after-ripening is presumably the result of slowing-down or distortion of metabolic processes of enzymatic and/or hormonal character, while the stimulation of softening with higher doses is probably the result of an increased permeability of cell membranes (SKOU, 1963) similar to the permeability changes and activation of polygalacturonase during the commencement of the physiological softening process in date tissue (COGGINS & KNAPP, 1969; HASEGAWA *et al.*, 1969). Therefore, the degradation of structural polysaccharides, first of all of pectin, is both caused and promoted by irradiation (SOMOGYI & ROMANI, 1964). These phenomena could compensate or even over-compensate the inhibitory effect of irradiation on the physiological softening processes. The initiation of after-ripening by increasing the permeability of the date tissue with the aid of freezing was reported in the early literature (RYGG, 1950).

The rather quick moisture loss during storage of fresh dates picked from their spikelets may be expected to influence the ripening processes and the growth of microorganisms, too, which further complicates the matter.

Under the experimental conditions applied, microbial spoilage occurred only in the *Lelwi* samples picked in khalaal stage (para. 2.3). The fruits picked from the same bunches in early rutab stage could be stored for a considerable time without moulding or fermentation in spite of the fact that their initial moisture content and hydrature was above the critical level for mould growth. [The 70—75% relative humidity, below which moulds are unable to grow, is reached at a moisture content of 24—30% (RYGG 1948; DOWSON & ATEN, 1962).] This difference in microbial stability of khalaal and rutab dates would be worth an exhaustive study with particular reference to the concentration and chemical composition of phenolic compounds in dates.

Microbial spoilage of khalaal *Lelwi* samples stored at 16—26°C was considerably reduced by irradiation with doses above 90 krad (para. 2.3, Table 6 and Fig. 16). Taking the time period to reach 5% spoilage as a practical measure of shelf life, the latter could be doubled by doses of 540 krad. Taking the shelf-life interval of untreated dates into consideration, the spoilage loss in the 540-krad samples amounted only to one-fifth of that of the untreated samples during the same time period.

The dibis yield from fully rutab dates (para. 2.5, Tables 7 and 8) was highly increased by radiation doses of 375 to 2 000 krad. This effect is in good agreement with the experiences on other fruits gathered in several laboratories, for example in the All-Union Scientific Research Institute for Canning and Vegetable Drying Industry, Moscow (FRUMKIN *et al.*, 1961; ROGACHEV, 1966)

and the Central Food Research Institute and the Research Institute for Viticulture, Budapest (FERENCZI & ÁSVÁNY, 1971) and it constitutes a promising possibility for utilization of ionizing radiation in dibis manufacture. The lighter colour and the significantly reduced viable counts of dibis (Tables 9, 10 and 12) can be considered as advantageous side effects of irradiation from the point of view of appearance and microbiological quality of the product. The increased dibis yield can be explained by the increased permeability of the irradiated date tissue and the radiation degradation of pectin as the stiffening agent of plant tissue present in the interlamellar space between individual cells. The softening of the date-flesh by irradiation, illustrated in Fig. 18, supports this explanation. The softening effect of irradiation was observed with many other fruits, too (BOYLE *et al.*, 1957; SOMOGYI & ROMANI, 1964; BRAMLAGE & COUEY, 1965).

On the basis of the exploratory experiment on radurization of diluted dibis (para. 2.5.6, Table 14, Fig. 19), the radiation preservation of dibis of high moisture content (semi-concentrated dibis of good sensory quality) seems to be possible. [It was reported by MRAK (1941) that the yeasts belonging to the *Zygosaccharomyces* genus grow in date syrup containing 66 per cent sugar. A blown can of commercial dibis of 66% refractometer value examined by the present authors showed CO_2 in the head-space and yeast ring at the borderline of dibis surface and the side wall.]

*

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DETERMINATION AND PRACTICAL APPLICATION OF THE THERMAL CONDUCTIVITY OF FLOURS

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An instrument was developed to measure the thermal conductivity of flour. The instrument and the technique of measurement is based on the principle of concentric spheres. The outer sphere was placed in a thermoregulated space. The material to be investigated was placed in the same sphere. The inner sphere, provided with heating and made of a material of high thermal conductivity, was placed in the geometrical center of the material to be tested. In the course of measurement the following parameters were established: thermo-flux, temperature of the thermostat, density of the sample to be tested and its grain distribution. In the knowledge of these data the thermal conductivity of the flour was established. It was found that thermal conductivity is strongly affected by temperature and density. The influence of moisture content and of grain size is negligible.

Knowing the thermal conductivity of flour an electric analogue model was constructed and used for the determination of the cooling characteristics of flour in silos constructed of reinforced concrete or in outdoor steel storage tanks. The electric analogue model is an RC unit of several storage compartments, where R is the reciprocal of the thermal conductivity of flour and C the heat content of the stored flour. In case of building a silo the thermal conductivity of the silo wall and its thermal capacity was also taken into consideration.

The development of the baking industry and, within its frame-work, up-to-date transportation and storage of flour confront the designer with a number of relevant factors, hitherto unknown. With increasing labour shortage and a requirement for more hygienic transportation of flour, bulk transportation is gaining ground.

Flour transported in bulk needs silos for storage, thus in newly built bakeries the construction of silos is necessary instead of storage space for sacks.

Storage facilities built of concrete and insulated, follow the fluctuations of temperature due to climatic factors relatively slowly, at the same time their building is expensive and time-consuming. They take about 2 years to dry out completely. Thus the construction of steel storage facilities seems simpler. It is a problem, however, whether the climatic conditions in Hungary permit the storage of flour under these conditions without its impairment. Is it necessary to provide an outdoor steel storage tank with insulation?

Problems of this kind made it desirable to determine instrumentally the heat conductivity coefficient of various types of flour of different moisture content.

TYE (1969) studied the heat conductivity of materials of different physical state, however, he did not report data related to foodstuffs. His data on heat conductivity as a function of temperature, density and grain size relate to diatomaceous earth and quartz sand. The effect of temperature is not unambiguous for every material. Heat conductivity in metals decreases with increasing temperature, while that of insulating materials increases.

GAUNITZ and co-workers (1969) report on the routine measurement of heat conductivity in fire-proof building materials. They use a heating filament determination method at high temperatures.

LENTZ (1961) determined the heat conductivity coefficient in meat, fat, gelatine gels and ice in the temperature range between +20°C and —20°C.

ROTH and co-workers (1970) measured the thermal conductivity of starch slurry. The data obtained were used to calculate and extrapolate the thermal conductivity of granular starch.

VYSELEVSKII and GROMOV (1967) discuss the thermophysical characteristics of flour. They critically evaluate a method described in several journals published in the Soviet Union, and find that the thermophysical characteristics are mostly influenced by density. It was established by them that with increasing density 1.5—2-fold, the value of λ increases 1.9-fold.

LAUBITZ (1959) studied the heat conductivity of granular powdered substances in the range between 100 and 1 000°C. He carried out the measurements in two stages. In the first stage he measured the λ value of the two phases: gas and solid particles, and in the second stage the equivalent radiant heat conductivity.

WOODAMS and NOWREY (1968) describe the heat conductivity coefficients of different foodstuffs and methods for the determination of the λ value of various materials, as given in the literature. There are four methods for the determination of the thermal conductivity coefficient:

1. the method of coaxial cylinders,
2. the method of concentric spheres,
3. the method of parallel plates,
4. the method of thermodiffusion.

In the first method two coaxial cylinders are used. Heating is provided in the inner cylinder and the outer one is submerged in a water bath to ensure the constant temperature of the outer wall. The material to be measured fills the space between the two cylinders. This method was used to determine the thermal conductivity of milk, buttermilk, edible oils, sugar solutions and of fruit juices.

The method based on the application of concentric spheres is of similar principle. The inner sphere has electric heating and the outer one is submerged in a water bath. The material to be investigated is placed between the two

spheres. This method was used to determine the heat conductivity of fats and grains.

The method of two parallel plates places the material between the two plates the upper one of which is provided with heating, the lower one with cooling. The heat conductivity of ice, human blood, milk, honey, egg-yolk, white of egg, frozen egg-white, various fats, filets of fish, fruits, vegetables and meats was determined with this method.

The thermodiffusion method is an indirect method of heat conductivity determination. In the knowledge of specific heat and specific density the thermal conductivity is determined by computation. The heat conductivity of orange, grapefruit and of various pulps was calculated by this method.

ALMÁSI (1958) used the method of concentric spheres to establish the heat conductivity of several foods. Both spheres were prepared of copper. The outer sphere was kept in a bath of constant temperature, while the temperature of the inner one was controlled with a copper-constantan thermoelement. The instrument developed by him was calibrated with a material of known thermal conductivity coefficient (water). He determined the thermal conductivity of the following food items: apple pulp, tomato puree, fruit cheese, green peas, ground paprika, beef, bacon and bones.

DANILOVA and co-workers (1959) used two methods, that of concentric spheres and that of coaxial cylinders, to determine the heat conductivity of flour. They kept the density of the sample at a constant value, while the temperature was varied. The experiments were extended over the measurement of the heat conductivity of paste. For the latter they used the method of coaxial cylinders.

MERSIOWSKY and ATHENSTADT (1972) report on their study carried out in an outdoor metal silo, as applied in the baking industry of the GDR. Several parallel heat sensors were placed radially in the silo and temperature changes in the flour during a 20-day storage period were recorded. The humidity of the air cushion above the flour and the moisture content of the flour were also measured. The eventual impairment of the flour during storage was controlled by technological tests.

1. Materials and methods

1.1. Flours tested

Flours marked BL 55, BL 80, BL 112, RL 56, RL 90 and RL 125 were tested. (BL = wheat flour; RL = rye flour. Numbers 55, 80, 112 and 56, 90, 125 designate flours of which the ash content, related to their solids content, is 0.55, 0.80, 1.12, 0.56, 0.90 and 1.25%, respectively.)

1.2. Determination of the thermal conductivity of flour

To determine the heat conductivity coefficient (λ) of flour, a measuring instrument based on the principle of concentric spheres was developed at the Institute. The theoretical principle of the circuitry is shown in Fig. 1.

The direct voltage required for measurement is provided by feed unit (*1*), as seen in the figure. Temperature is controlled with the high accuracy regulator SKI HPt (*2*), designed and prepared at this Institute. The sensor of the temperature regulator is a Pt resistance thermometer (*3*). The continu-

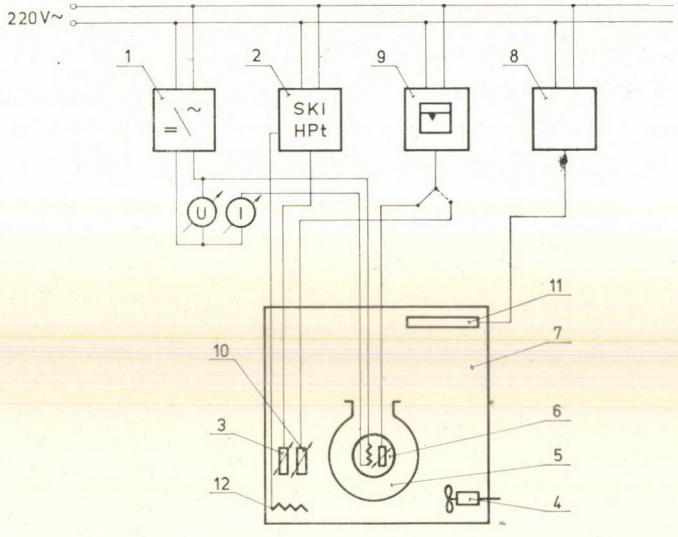

Fig. 1. Theoretical connexion diagramme of an instrument for the determination of thermal conductivity (for legend see text); U — Voltmeter; I — power meter

ous air circulation in the closed space is ensured by ventilator (*4*). The test material is placed in sphere (*5*) and in the geometrical center of the outer sphere is the inner sphere (*6*) made of high conductivity metal and provided with electric heating. The measurement is carried out in a closed and highly accurate temperature thermostat (*7*). For measurements at low temperatures a cooling device (*8*) is provided. Temperature is registered throughout (*9*). To be able to control the actual temperature in the closed space Pt thermometer (*10*) was built in the thermostat. The evaporator (*11*) of the refrigerating device and the heater (*12*) were also built in the thermostat.

Prior to a series of measurements the volume of the sphere containing the test material or its geometrical dimensions and the diameter of the heated sphere were established. The ratio of the two diameters serves as constant in the calculations. The moisture content of the flour samples was also determined prior to placing them in the sphere. (To determine the moisture content

a test portion of the sample was dried for 2 hours at 130°C in a drying oven.) The sample was then introduced into the sphere of known volume. In the knowledge of the weight of flour introduced the density of the flour could be calculated. The sphere with the sample was then placed in the thermostat and the desired temperature was set on the control device. The air circulation was set into operation and the heating voltage required to the measurement was also set. The change in temperature of the inner sphere with time was read off the temperature recorder. About 24 hours were required for the equilibrium to set in. Then the temperature of the inner sphere and the heating capacity were established with great accuracy. In the knowledge of the thermostat temperature the difference between the two temperatures gave the Δt value required to carry out calculations.

The λ value, or in other words the thermal conductivity of a flour sample of given quality, moisture content, density and temperature, was calculated from the temperature, the heating capacity and the geometrical parameters.

Hereafter the temperature of the thermostat was raised by 10°C and the above measurements were repeated after the lapse of another 24 hours. Thus a second value was obtained. The thermal conductivity of the flour was measured at 10°C intervals.

A flour of given moisture content and density was tested at five different temperatures. This series of measurements were carried out at three different moisture contents.

The calculation of the thermal conductivity of flours followed the pattern shown below. The value of heat flux in the case of concentric spheres is:

$$\Phi = \frac{2\pi\lambda}{\frac{1}{d_b} - \frac{1}{d_k}} (t_b - t_k) \quad [W],$$

$$\lambda = \frac{\Phi \left(\frac{1}{d_b} - \frac{1}{d_k} \right)}{2\pi (t_b - t_k)} \quad [W \cdot m^{-1} \cdot K^{-1}],$$

where:

Φ = heat flux (W)
λ = thermal conductivity coefficient ($W \cdot m^{-1} \cdot K^{-1}$)
d_b = diameter of the metal sphere provided with electric heating (m)
d_k = diameter of the sphere containing the sample (m)
t_b = temperature of the heated sphere (K)
t_k = temperature of the thermostated space (K).

Using the constant deduced from the geometrical parameters

$$\lambda = C\frac{\Phi}{\Delta t} \quad [W \cdot m^{-1} \cdot K^{-1}] .$$

The value of heat flux was established on the basis of the electric heating capacity.

1.3. Development of the electric analogue model

In the knowledge of the thermal conductivity of flour, as determined by the above method, an electric analogue model was developed and used for the determination of temperature in flour at various places of a silo in case of industrial flour storage. The R value of the four compartment RC member analogue model is the reciprocal of the thermal conductivity of the flour, while the C value is the heat content of the flour. The connection diagramme of the analogue model is given in Fig. 2.

The electric analogue model represents a steel silo in which the ratio of the volume of selected flour layers, and, thus, of their heat capacity is 1 : 3 : 5 : 7. Similarly, the ratio of built-in condensers is also 1 : 3 : 5 : 7. In the case of steel silo walls the thermal conductivity coefficient of the wall is higher by several orders of magnitude than that of the flour, thus member R is negligible. However, in the case of concrete silos the thermal conductivity of the walls is highly dependent on the insulation, thus, as seen in the con-

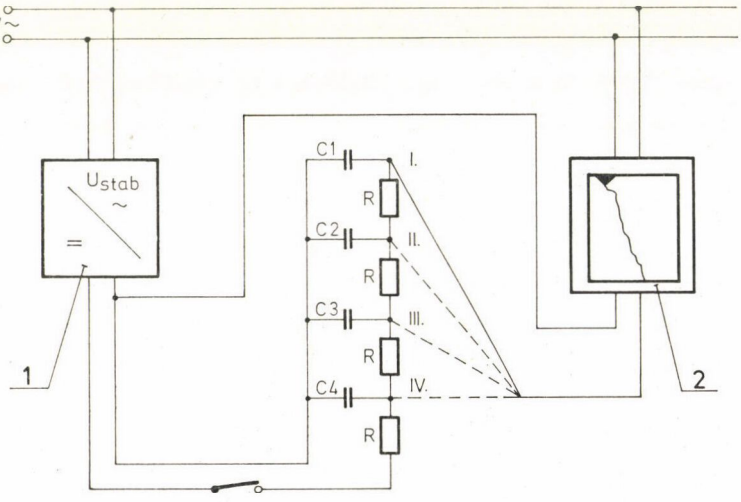

Fig. 2. Analogue model for steel storage silos. 1 — Stabilized feed voltage; 2 — Recorder; R — 10 kΩ resistance; C_1 — 100 μF; C_2 — 300 μF; C_3 — 500 μF; C_4 — 700 μF. Symbols I—IV are explained in Fig. 6

nection diagram in Fig. 3, the reciprocal of the thermal conductivity and the heat capacity of the wall was also included.

Constant voltage was used in the analogue model.

The cooling diagrammes of flour, as established with the analogue model, were controlled under winterly climatic conditions in practical flour storage tests. A steel walled flour silo of 10 t capacity was set up at the Institute and

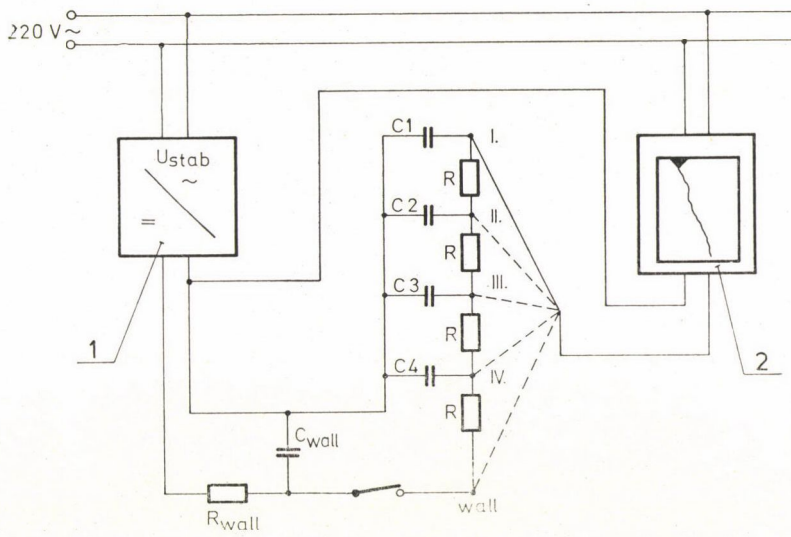

Fig. 3. Analogue model for a silo built of concrete. 1 — Stabilized feed voltage; 2 — Recorder; C_1 — 100 μF; C_2 — 300 μF; C_3 — 500 μF; C_4 — 700 μF; R — 10 kΩ resistance; C_{wall} — Heat capacity of the wall; R_{wall} — Heat resistance of the wall. Symbols I—IV are explained in Fig. 6

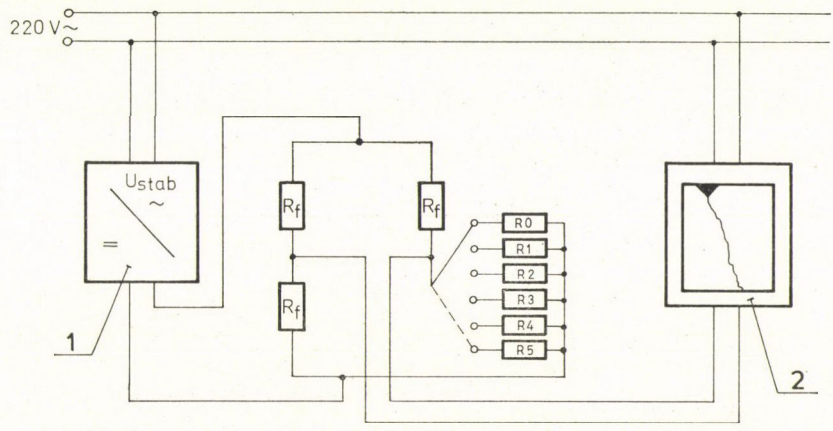

Fig. 4. Theoretical connexion diagramme of a temperature recorder placed in an outdoor silo. 1 — Stabilized feed voltage; 2 — Recorder; R_f — Constant resistance; $R_0 - R_5$ — Pt resistance thermometer

this was fitted in radial direction with 5 Pt resistance thermometers. The cooling of the flour was measured and recorded during an 11-day period, using the connection scheme as shown in Fig. 4.

The ambient temperature was simultaneously recorded.

2. Results

2.1. Determination of the heat conductivity coefficient

With the apparatus described on the basis of Fig. 1, measurements were carried out on six flour varieties at three different moisture contents and at five different degrees of temperature. On plotting the results the correlation λ–temperature–density was found linear. As seen in Fig. 5, with increasing temperature and density, the value of λ increased.

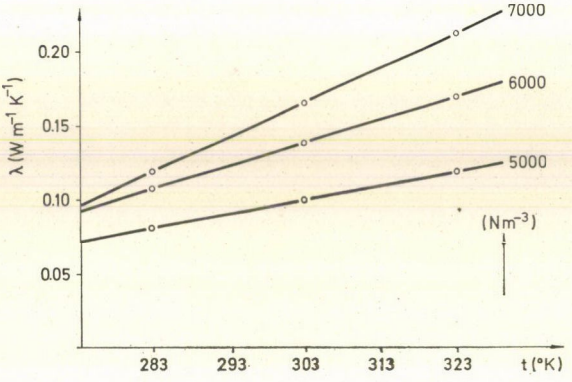

Fig. 5. Heat conductivity coefficient (λ) of flours as a function of temperature (t) and density ($N \cdot m^{-3}$)

In order to find out the influence of grain size distribution and of the moisture content of flour upon the thermal conductivity coefficient of flour the results of the measurements were analysed according to various variables. It was found that in the range investigated the value was not significantly affected by either grain size distribution or moisture content. The thermal conductivity is highly dependent only on temperature and density. The λ value is not affected by the type of flour either.

The equations of the curves based on the points of measurements are as follows:

$$\lambda_{5\,000\ N \cdot m^{-3}} = -0.18174 + 0.00093t \quad W \cdot m^{-1} \cdot K^{-1}$$
$$\lambda_{6\,000\ N \cdot m^{-3}} = -0.32429 + 0.00153t \quad W \cdot m^{-1} \cdot K^{-1}$$
$$\lambda_{7\,000\ N \cdot m^{-3}} = -0.53746 + 0.00232t \quad W \cdot m^{-1} \cdot K^{-1}$$

2.2. Experiences gained with the electric analogue model

Based on the connections, as seen in Fig. 2, the curves showing the cooling of flour in a steel-walled silo are marked I, II, III, IV in Fig. 6.

The Δt temperature differences between the temperature of the stored flour and the environmental temperature are indicated on the vertical axis (fluctuations in the environmental temperature being ignored). On the horizontal axis the time is indicated. Curve marked IV indicates the temperature of the flour layer next to the wall, curves marked I, II and III belong to layers inside the silo.

When conditions other than those prevailing in steel silos are imitated with the analogue model, the resulting curves, based on the connection diagramme as shown in Fig. 3, follow the pattern according to Fig. 7.

The layer of heat insulation in the wall is warmed by the flour and reduces the rate of cooling of the flour within the silo.

2.3. Results of practical flour storage studies

The steel storage tank of 10 ton capacity, set up at the Institute was used for extended storage experiments during winter. The cooling of the flour was followed by 5 radially placed Pt thermometers, interspaced 250 mm. The measurements and recording were carried out according to the connection diagramme in Fig. 4. Results are shown in Fig. 8.

It is evident from the figure that the layer next to the wall follows relatively promptly the climatic temperature fluctuations. However, at a distance of 250 mm from the wall the fluctuations are hardly noticeable in the temperature of the flour. In layers more distant from the wall, changes due to temperature fluctuations are not perceptible.

3. Conclusions

3.1. Determination of the thermal conductivity coefficient of flour

The thermal conductivity coefficient of various kinds of flour, of varied moisture content and grain size distribution, was determined at five different temperatures and three different density values with the apparatus shown in Fig. 1. In the knowledge of the measured data the thermal conductivity curves were plotted as a function of density and temperature. It was found that with increasing density or temperature, the thermal conductivity coefficient of flour increased.

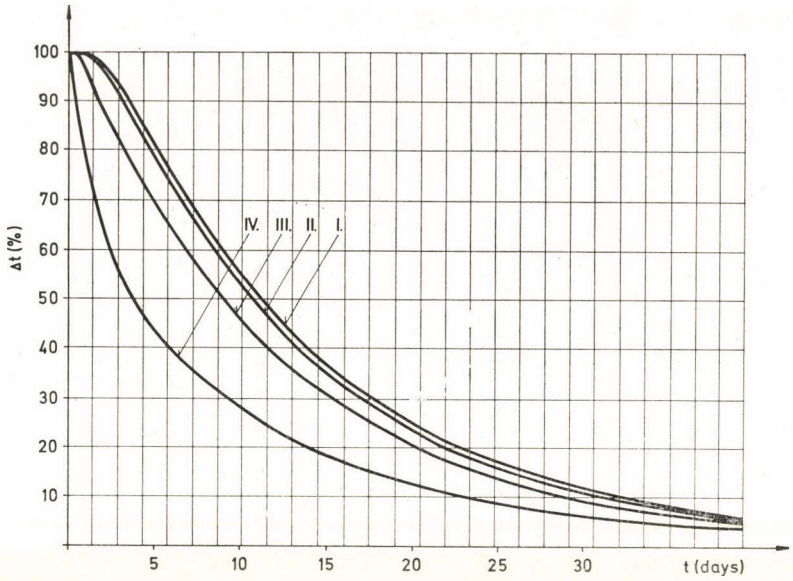

Fig. 6. Analogue model. Cooling curves of the different layers of flour fed into the silo as a function of time. "IV" cooling curve of the layer next to the wall; "III" cooling curve of the layer at 250 mm from the wall; "II" cooling curve of the layer at 500 mm from the wall; "I" cooling curve of the layer at 750 mm from the wall

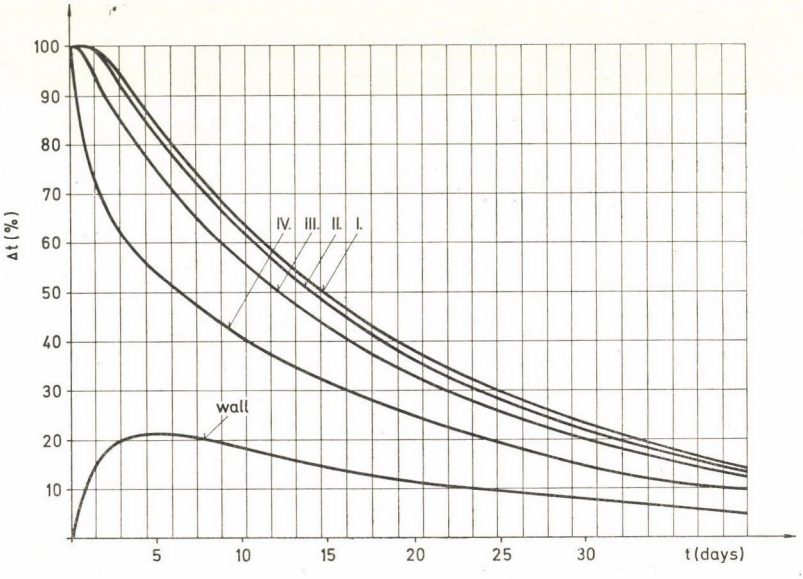

Fig. 7. Analogue model. Cooling curves of the different layers in a silo built of concrete as a function of time. Symbols I—IV are the same as in Fig. 6

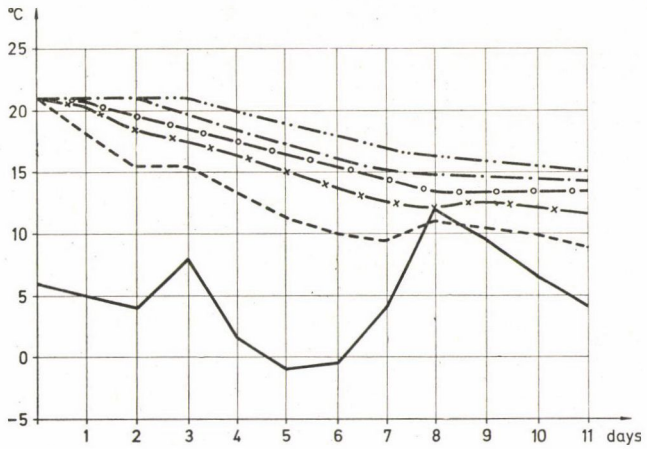

Fig. 8. Cooling curve of flour in an outdoor silo. ——— Average ambient temperature; —·—·— Thermometer 0; —·—·— Thermometer 1; —○—○— Thermometer 2; —×—×— Thermometer 3; - - - - - Thermometer 4

On comparing the results of measurements with the values as seen in Fig. 5, giving the thermal conductivity coefficient as a function of density and temperature, the standard deviation was found as follows:

$$s = 0.008886 \ W \cdot m^{-1} \cdot K^{-1}.$$

This shows that in possession of Fig. 5 the knowledge of only the density value is necessary to obtain thermal conductivity values satisfying practical requirements. As seen in Fig. 5, the thermal conductivity coefficient values of flour as a function of temperature and density fall between

$$\lambda = 0.0722 \ W \cdot m^{-1} \cdot K^{-1} \quad \text{and} \quad \lambda = 0.2119 \ W \cdot m^{-1} \cdot K^{-1}$$

(the density values of the samples were between 5 000 and 7 000 $N \cdot m^{-3}$).

Results obtained in this study were compared to those reported by VYSELEVSKII and GROMOV (1967) on flours having thermal conductivity coefficients between $\lambda = 0.1163$ and $\lambda = 0.1396 \ W \cdot m^{-1} \cdot K^{-1}$, and to those of WOODAMS and NOWREY (1968) who studied wheat of $\lambda = 0.1500 \ W \cdot m^{-1} \cdot K^{-1}$ thermal conductivity coefficient at 7 800 $N \cdot m^{-3}$ density, further to those of CHUBIK and MASLOV (1970), having obtained values between $\lambda = 0.112$ and $\lambda = 0.130 \ W \cdot m^{-1} \cdot K^{-1}$ in a flour of 7 620 $N \cdot m^{-3}$ at temperatures in the range of —5°C to +35°C. The experimental data were in good agreement with those found in the literature.

3.2. Conclusions drawn from work with the analogue model

With the analogue model developed, knowledge of the thermal conductivity coefficient of flour makes it possible to determine the temperature of flour layers as a function of time, both in steel silos, or silos built of concrete and insulated.

It was established in experimental storage and with the aid of the analogue model that using a steel tank insulated with a material of low conductivity, for instance cork of $\lambda = 0.0348—0.0465$ thermal conductivity coefficient, applied in a layer of appr. 120 mm thickness, the temperature of the flour, after 11 days storage, would be about 2 degrees higher than in the same storage facility without insulation (under winter conditions). Thus it may be concluded that under the climatic conditions prevailing in Hungary the insulation of steel tanks is superfluous. (Mean temperatures for the three winter months as averages of the last thirty years: December: +1.2°C, January: —1.3°C, February: +1.2°C.)

An undesirable phenomenon sometimes observed in bulk storage of flour is the pastiness of the layer in direct contact with the wall of the tank. Dew formation on the wall of the tank occurs, when in an empty tank the inside air is relatively warm, while the temperature of the environmental air rapidly decreases. In this case the wall of the tank cools down as well, and dew formation begins on its inner surface. If at the same time flour is infed, the flour, on contacting the wet wall, becomes pasty. To prevent the occurrence of this process it is advisable to thoroughly aerate the tank prior to filling, thereby cooling the air inside the tank and preventing dew formation.

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STUDY INTO THE APPLICABILITY OF ISOLATION METHODS AND INJECTION INTO A CHROMATOGRAPHIC COLUMN OF VOLATILE PRODUCTS OF MAILLARD'S REACTION IN MODEL SYSTEMS

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It is known that the method of isolation and introduction of samples of odour substances from food affects to a great extent their separation by GLC and the consistence of the chromatographic pattern with the composition of the substances in the material under study.

The present study was designed to select a method of separating, and injecting into the GLC column, volatile Maillard reaction products obtained from a model reaction between amino acids and sugars. For this purpose a number of methods selected from literature were tested.

It was concluded that separation of these compounds in a neutral gas flow and subsequent condensation in the precolumn on a neutral carrier appeared the best. The injection of the sample by means of a two-way valve improves the reproducibility of the results. The method developed for separating and introducing compounds into the gas chromatograph permits the analysis of substances in a wide range of boiling temperatures.

In the course of studies on volatile odour substances — products of Maillard's reaction in model systems — the authors faced some difficulties in selecting a method for their isolation as well as injection into a column of a gas chromatograph for further analysis.

It is known that the way of isolating and injecting a sample of odour substances from food into a chromatographic column affects to a great extent their distribution, and the reproducibility of the results obtained, as well as the consistence of the chromatographic pattern with the actual composition of substances in the material under study.

Nowadays, a number of methods are applied to the isolation, concentration and injection into a gas chromatograph of odour compounds from food products, and the nature of the products to be tested determines the method to be used.

Thus the following methods are in common use:

1. Extraction of odour substances with a solvent and injection by means of a syringe as used, *e.g.* in separating odour compounds from pork, by Hornstein and Crowe (1960), from bread by Hunter and co-workers (1961) and in isolating odour compounds from pears by Jennings (1961). This method is useful in separating substances of relatively high boiling temperature (higher than the boiling temperature of the solvent).

2. A distillation method connected with extraction, such as, for instance, the one used by BRANDENBERGER and MÜLLER (1962) in isolating odour compounds of tea as well as by HERZ and CHANG (1966) in separating odour compounds from beef — likewise applicable for isolating substances of relatively high boiling temperatures.

3. High-vacuum distillation connected with cold trapping in liquid nitrogen as used among others by MERRITT and co-workers (1959) in isolating odour compounds from irradiated beef. This method permits to recover, in fact, all the odour compounds, however, with mixtures containing compounds of very high boiling temperatures injection into the chromatograph presents difficulties.

4. A method consisting in the separation of substances in a neutral gas flow and (*a*) cold trapping in liquid nitrogen as used by KRAMLICH and PEARSON (1960) in the separation of volatile compounds from boiled beef or (*b*) retaining the separated compounds in a neutral odourless solvent of high boiling temperature (as described by NONAKA, 1971).

5. A method of separating some groups of compounds through transformation into stable derivatives as a result of reactions with group reagents:

(a) carbonyl compounds (RALLS, 1960; BUTTERY, 1961; LINKO *et al.*, 1962);

(b) sulphur compounds (CHALLENGER and CHARLTON, 1947);

(c) amines (JAMES *et al.*, 1952; BURKS *et al.*, 1959).

Stable derivatives can be regenerated to initial compounds before injection into the chromatographic column. In the analysis carried out by means of any of the above mentioned methods, the fact that odour compounds occur in food products in very small quantities, caused another difficulty. Since losses or changes in those compounds take place mainly during their isolation, the selection of an appropriate method is particularly important. Mistakes made in this phase cannot be corrected later on. One has to be especially careful about the protection against any unnecessary pollution or artefacts. This study was designed to select a method of separating and injecting into the column of the gas chromatograph volatile products of Maillard's reaction as obtained from model reactions between amino acids and sugars.

Since the products of the reaction between amino acids and sugars represent a very wide range of boiling temperatures (from —62°C for H_2S to above 180°C for certain hydrocarbons) their complete separation and injection into the chromatograph may prove the correctness of the method used.

For this purpose a number of known methods that seemed suitable for separating volatile products of the reaction between amino acids and sugars have been tested along with trials to introduce various modifications developed in this laboratory. It was hoped that these modifications would (a) provide

the most complete separation of these compounds as well as (b) solve the problem of their injection into the chromatographic column without appreciable losses.

1. Materials and methods

The study was carried out with products of the reaction between methionine and glucose.

1.1. Reagents

Methionine, *Reanal*, analytical grade, and glucose, *Poch*, analytical grade.

1.2. Conditions of the reaction

The reaction was performed in an aqueous solution at pH 6.5—7 in a closed system, in a reactor of 50 ml capacity, at a temperature of 120°C during 60 min. Reagents were used in equimolar concentrations amounting to 105 μmole · ml^{-1}. The methods described by HERZ and CHANG (1966) as well as by NONAKA (1971) were used for the separation of volatile reaction products. After separating by various methods and injecting into the chromatograph, reaction products were separated under the following conditions: apparatus: JEOL JGC-810, column: length 3 m, diameter 3 mm, filling: Chromosorb G DMCS 80—100 mesh, 5% DC 550. Detector: FID, detector temperature 210°C, injection temperature 195°C, sensitivity 1×10^2, tape speed: 0.5 inch · min^{-1}, gas flow: N_2 — 60 ml · min^{-1}, H_2 — 30 ml · min^{-1}, air — 2 atm. gauge pressure. Temperature programme of the column: isothermal operation at 40°C for the first 3 min followed by an increase in temperature up to 140°C at the rate of 4°C · min^{-1} and concluded by a 15 min isothermal period at 140°C.

1.3. Experiments

The first method used for the separation of volatile compounds was the one suggested by HERZ and CHANG (1966). This method comprises vacuum distillation and extraction in a liquid-liquid system. The apparatus was composed of two parts: one in which the sample to be tested was in aqueous solution and the other under vacuum with a number of cold traps. Cold trapped

compounds (diluted with water) were extracted by means of ethyl ether in a liquid-liquid system, and then concentrated by evaporation of the solvent. In the case of the substance under investigation this method caused a number of insuperable difficulties as follows:

(a) Considerable loss of lower boiling compounds occurring mainly during extraction with ethyl ether,

(b) low concentration of compounds in ether made the analysis by direct injection impossible, and during evaporation of the ether practically all the previously separated compounds volatilized.

Later on experiments to separate the volatile products of the reaction of methionine with glucose were carried out using the method suggested by NONAKA (1971) and the separated compounds were collected in dibutyl phthalate. The apparatus (Fig. 1) was composed only of a reactor (Fig. 2), a reflux connected with a U-tube filled with a drying agent and a receptor vessel of the shape of a washing bottle filled to 1/5 of its volume with dibutyl phthalate (not shown). This receptor vessel was cooled with ice water.

Since the trials as to the injection into the chromatographic column of the compounds dissolved in dibutyl phthalate did not give positive results (because of excessive dilution and blocking of chromatographic column flow by phthalate) it was decided to separate compounds dissolved in this solvent using vacuum distillation (as suggested by NONAKA, 1971). Experiment of vacuum distillation coupled with cold trapping of the distilled compounds, in order to improve chromatographic results, has also failed. The reaction products cold-trapped in liquid nitrogen rapidly expanded in the receiving vessel on its removal from liquid nitrogen, and led to its explosion. For safety reasons, further trials of separating the compounds dissolved in dibutyl phthalate by this method were given up.

In view of the above it was decided to use the system with a precolumn element 9 (Fig. 1), *i.e.*, with a 50 cm long and 3 mm ∅ stainless steel tube filled with a neutral carrier with moderately developed surface. For this purpose, 80 mesh BDH glass beads were used.

During the experiments, the precolumn was cooled using the following mixtures:

NaCl—ice (—20°C);
$CaCl_2 \cdot 6H_2O$—ice (—40°C);
Solid CO_2—acetone (—70°C);
Solid CO_2—ethanol (—70°C);
Liquid nitrogen (—196°C).

Following the isolation, the precolumn was disconnected from the system and connected with the gas chromatograph by means of a valve (Fig. 3). Then the compounds were injected into the gas chromatograph by means of desorption in an oil bath at 125—130°C during 3 minutes.

A successive experiment was aimed at the use of washing bottles filled with group reagents such as DNPH (2 g DNPH in 1 litre 2*N* HCl) in order to

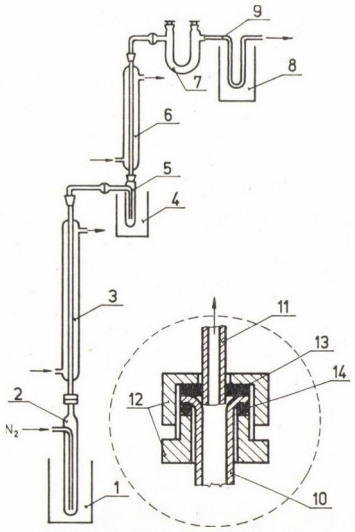

Fig. 1. Apparatus for isolation, fractionation and collection of volatile products from the model system. 1 & 4 — electric water bath; 2 — reactor vessel; 3 & 6 — condenser; 5 — trap with group reagent; 7 — U-tube with drying agent; 8 — solid CO_2–ethanol bath; 9 — precolumn; 10 — neck of reaction vessel; 11 — condenser neck; 12 — metal head; 13 & 14 — sealing O-rings. The insert depicts constructional details of the element connecting elements 2 and 3 of the main part of the figure

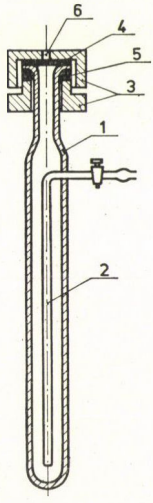

Fig. 2. Reaction vessel for the reaction and isolation of volatile products of the reaction between amino acid and sugar in a model system. 1 — reaction vessel; 2 — pipe for carrier gas; 3 — metal head; 4 — septum; 5 — sealing ring; 6 — hole of 2 mm ∅

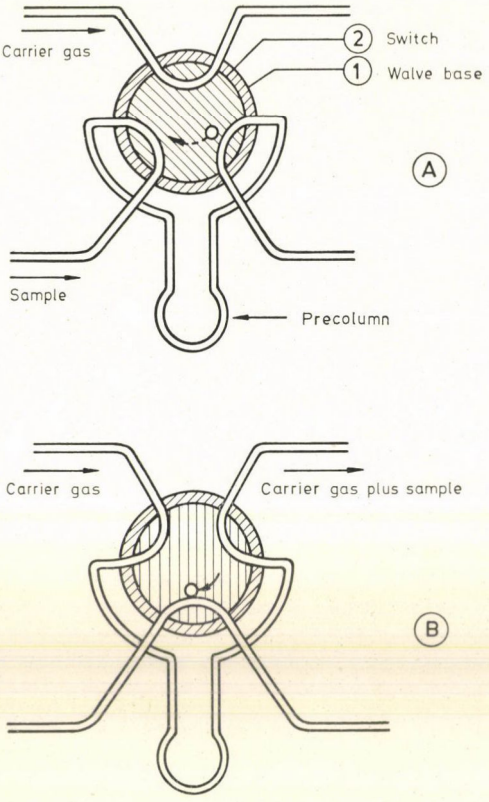

Fig. 3. Introduction of volatile samples to the GLC with a 2-way valve. A — collection of the sample on the precolumn. B — introduction of the sample on the GLC column

bind carbonyl compounds, $Hg(CN)_2$ (4% aqueous solution) to bind mercaptane and $HgCl_2$ (3% aqueous solution) to bind sulphides and disulphides.

These bottles were mounted between the reactor and the precolumn (Fig. 1).

2. Results

The method of HERZ and CHANG (1966) used at the beginning of the study was not suitable to separate volatile reaction products of amino acids and sugars. Its inexpediency was mainly due to strong dilution with water of the separated reaction products and insuperable difficulties during solvent extraction of the volatile products from the dilute aqueous solution. The NONAKA (1971) method seemed to be more suitable in this case. By this method, separation was carried out under very mild conditions, and owing to

the use of a neutral gas in continuous flow as extracting agent, the ratios of the components were kept similar to those in the post-reaction mixture. This is proven, among others, by the wide spectrum of boiling temperatures of the separated substances (from —62 to 180°C).

Due to the mild conditions and the neutral gas used in the separation of the compounds, the possibility of artefact production was undoubtedly limited.

As it appeared, without modifications this method was also unserviceable in the chromatographic analysis since the concentration of many volatile

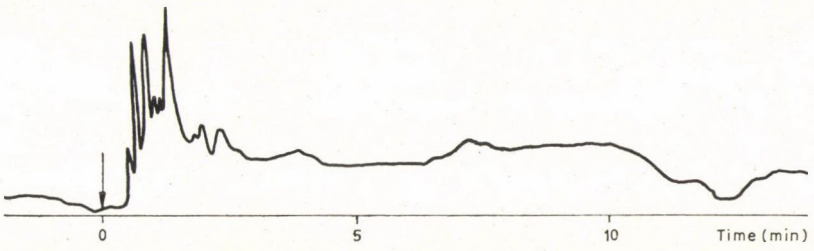

Fig. 4. Chromatogram of volatile methionine–glucose reaction products injected in dibutyl phthalate solvent

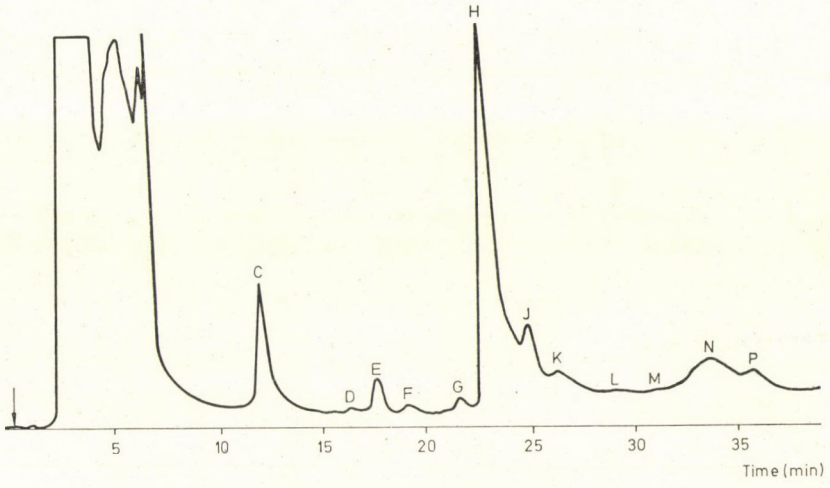

Fig. 5. Chromatogram of volatile methionine–glucose reaction products in a model system

substances dissolved in dibutyl phthalate was still beneath the sensitivity limit of the chromatographic method (compare Fig. 4).

Solely the use of a precolumn filled with an appropriate carrier, as a "trap", holding the separated compounds, as well as the use of a special valve, facilitated the injection of these substances into the chromatographic column and made analysis possible. The chromatogram of the volatile reac-

tion products of methionine and glucose, obtained under the above conditions as presented in Fig. 5, is characterized by a great accumulation of compounds in the first part of the chromatogram. This confirms the extraction of a large number of substances of low boiling temperatures. It should be stressed that the separation of the accumulated compounds which appear first in the chromatogram is impossible on the column of the given type. (These compounds were well separated on a column filled with Carbowax 1000, as used in the continuation of this study. The results of that part

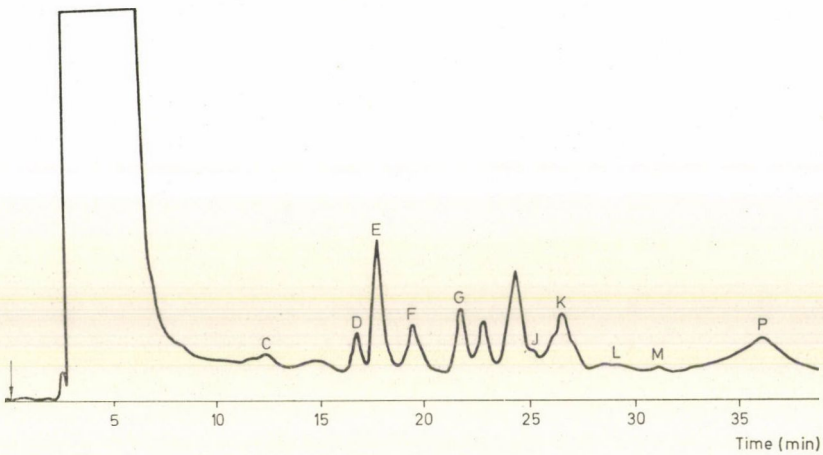

Fig. 6. Chromatogram of volatile methionine–glucose reaction products in a model system. [Mercaptans were trapped with $Hg(CN)_2$]

of the investigations will be presented in a separate paper.) However, a relatively good separation of the compounds with higher boiling temperatures was obtained which appear in the further parts of the chromatogram: numerous and high peaks (*e.g.* C, H, I, K) prove efficient in the separation and binding of the compounds of higher boiling temperatures on the precolumn.

The precolumn method increased the reproducibility of the results provided the conditions of chromatographic analysis were carefully observed. After injecting the sample into the chromatographic column, the precolumn could be easily separated from the valve (through which the compounds adsorbed on the carrier had been injected), and after emptying, washing and drying used again for another analysis.

The types of the cooling baths used, as well as rate and duration of carrier gas flow determined the number and type of compounds bound on the precolumn. The best results were obtained using a CO_2–ethanol bath for

precolumn cooling and, for the separation of volatile compounds from the system, nitrogen flow at a rate of 30 ml · min^{-1} for 1.5 hours.

The use of the precolumn involves, however, the danger of deterioration of the chromatographic separation due to the introduction of additional dead volumes which cause tailing and probable adsorption of compounds especially of higher boiling temperatures. These difficulties could be overcome by applying the shortest connection of the injection system (among others the valve) with the column, by lining these connections with deactivators (*e.g.* DMCS) as well as by heating.

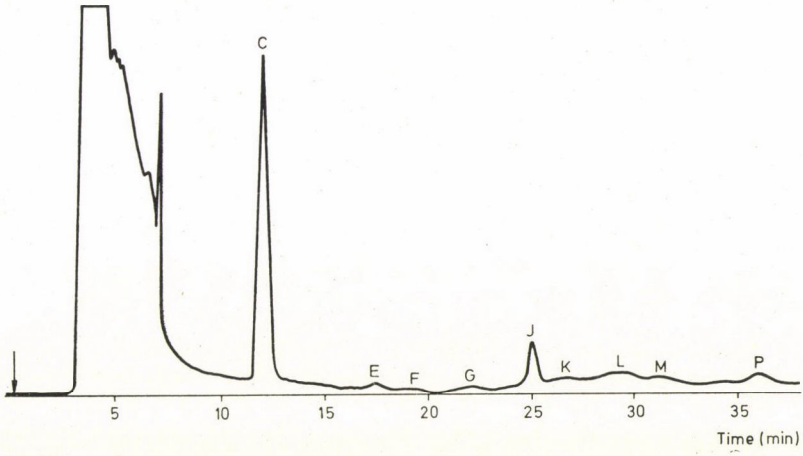

Fig. 7. Chromatogram of volatile methionine–glucose reaction products in a model system (sulphides and disulphides were trapped with $HgCl_2$)

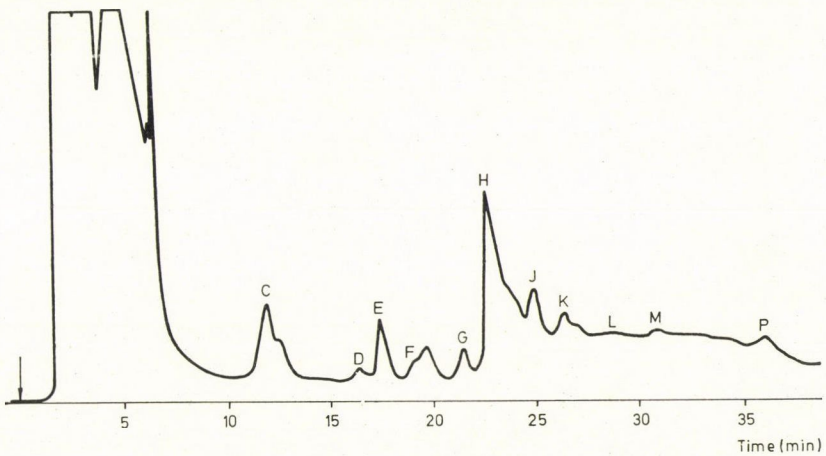

Fig. 8. Chromatogram of volatile methionine–glucose reaction products in a model system (carbonyl compounds were trapped with DNPH)

Utilizing, in the above mentioned system of separation and injection washing bottles with group reagents facilitates the qualitative interpretation of chromatograms and improves the separation of compounds through the elimination of their individual groups. For example, a relatively high peak, specified as C on the chromatogram in Fig. 5 was eliminated through the washing bottle containing a mercuric cyanide solution (compare Fig. 6), while the peak specified as H in Fig. 5 disappeared after using the washing bottle with $HgCl_2$ (compare Fig. 7) as well as with $Hg(CN)_2$. This suggests that the substance in question is a double-function compound containing both —S— and —SH groups.

Some peaks appearing on chromatography (Fig. 5) and specified as D, E, F, G, N, P increased after using washing bottles with $HgCl_2$. It may be assumed that these could be compounds originating from the water used in preparing the group reagents (probably of sulphide character).

When washing bottles with DNPH were used all peaks remained, starting from C; this may suggest the lack of carbonyl compounds in the mixture analysed (Fig. 8).

Some important methodological questions, which, when solved, improved the process of separation and injection into the chromatograph of volatile reaction products of methionine and glucose, involved a number of modifications of the NONAKA method as elaborated by the authors and applied in practice. These covered among others the elaboration and construction of a reactor (Fig. 2) which facilitated:

(a) the reaction of amino acids with sugars in an atmosphere of neutral gas,
(b) the collection, directly after the reaction, of volatile products from head space (without contamination) in order to carry on the chromatographic analysis using the "head space" method,
(c) the connection (without the necessity of removing the post-reaction mixture to another vessel) with the separation system of volatile compounds,
(d) the use of a receptor vessel with dibutyl phthalate,
(e) the use of washing bottles with group reagents.

A reactor of this type may be used for separating flavour substances from various types of homogenates of foodstuffs.

The necessity of very careful purification of water, drying agents, carrier gas and apparatus used throughout the experiments presents another problem and causes great difficulty in the methods of separating flavour compounds, as any impurity coming from those sources accumulates on the precolumn (or another type of receiving vessel) falsifying the chromatographic pattern.

The chromatograms of volatile compounds separated from distilled and redistilled water after discarding the forerun (Fig. 9) may serve as an example.

As it appears from the chromatograms presented, even repeated distillation did not purify water completely, however, repeated distillation and

discarding of the forerun containing most of the impurities may be considered as efficient.

In order to eliminate impurities the following purification was applied:

(a) water, by redistillation and discarding the forerun; the rest was used for preparing reagents and solutions,

(b) calcium chloride and other compounds, by vacuum drying (below 1 mm Hg) at 80°C,

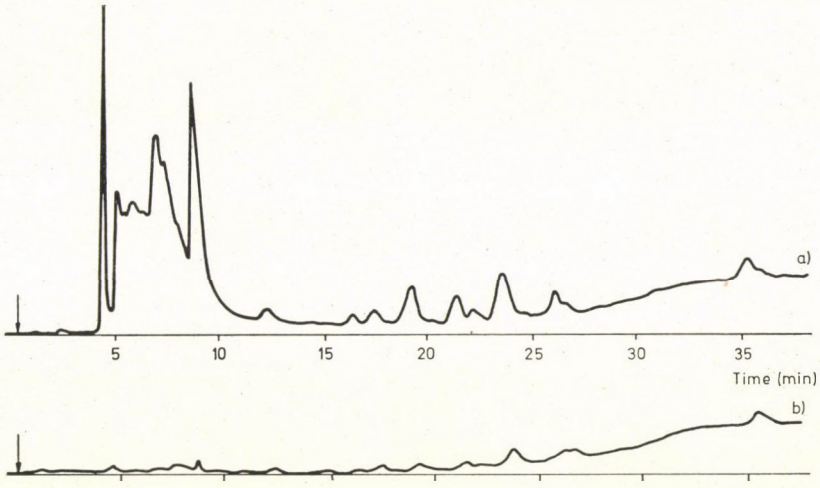

Fig. 9. Chromatogram of volatile comounds separated from: a) distilled and b) redistilled water after discarding the forerun

(c) glass beads and glass wool, by concentrated HCl treatment for 24 hours, then washing with redistilled water and vacuum drying,

(d) carrier gas, filtered through a molecular sieve, type 5A and silicagel; (gas was conveyed to the system by teflon tubings),

(e) glass apparatus (all ground joints) washed with detergents and water, rinsed with ethyl alcohol and dried at 120°C.

The analysis was preceded by the blank sample (without reagents) in order to be sure that the whole system was completely pure.

3. Conclusions

Among the tested methods (and their modifications) of separation and injection into a gas chromatograph of volatile products of Maillard's reaction in model systems, the method of separation of these compounds in a neutral gas flow with their condensation on a neutral carrier with moderately developed surface appeared to be the best one.

The injection of a sample from the precolumn by means of a special valve improves the reproducibility of the results.

The method used for separation and injection of compounds makes possible the introduction of compounds of a wide spectrum of boiling temperatures into a gas chromatograph.

In connection with the above this method may be widely used in separation and injection into a gas chromatograph of odour substances from food products. The application of washing vessels with group reagents may additionally facilitate the qualitative interpretation of chromatograms.

*

The authors would like to thank Dr. Nina BARYŁKO-PIKIELNA for her assistance and advice given in the course of the study and in the elaboration of results, as well as Elzbieta FILIPIAK for helpfully performing the experiments.

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DISPERSOID-ANALYTICAL STUDIES ON COMMINUTION OF FOOD SUSPENSIONS

II. EXPERIMENTS WITH COMMINUTORS BASED ON MECHANICAL EFFECT

GY. URBÁNYI

(Revised manuscript received May 7, 1973)

Quince, plum, apricot and tomato suspensions were used in comminution experiments carried out with two comminutors based on the mechanical principle: 1. MSE homogenizer; 2. Fryma mill with corundum discs.

The experimental material was reduced to various degrees of fineness. The suspensions thus obtained were subjected to dispersoid-analytical examination using the sedimentation technique elaborated earlier by the author.

Experiments with the MSE homogenizer have shown that the most extensive particle size reduction took place in the initial phase of comminution. Suspensions of originally rougher distribution suffered more extensive reduction than those of finer distribution.

In experiments with the Fryma mill the effect of the distance between the corundum discs and of their granulation on particle size distribution was studied. It was found that for effective comminution a smaller distance is required between discs of finer granulation.

The maximal comminuting effects were characterized by the C_{25}, C_{50} and C_{75} values representing the quotients of the lower quartile, median and upper quartiles of the untreated suspension and the maximally treated suspension.

The C-values calculated for the comminutors to be tested were represented as a function of the corresponding parameters of the original suspensions, using the results obtained with the two ultrasonic comminutors described in the first part of this study.

As experiments have shown, the highest comminuting efficiency was achieved with the MSE homogenizer and the Minisonic apparatus described in the first part of this study.

By determining the functions between the C values and the parameters and solving them for $C_{25} = 1$, $C_{50} = 1$ and $C_{75} = 1$, respectively, the parameters of each comminutor were established for the fibre suspension in state of agglomeration equilibrium. The average values belonging to the parameters are as follows:

Lower quartile (Q_1):	5 μm
Median (Md):	16 μm
Upper quartile (Q_3):	41 μm.

Suspensions having such parameters, containing plant cell constituents dispersed in distilled water, cannot be further comminuted by equipment used in the experiments or working on similar principles, under similar conditions.

In an earlier paper of the author (URBÁNYI, 1972) the results of experiments carried out with two ultrasonic comminutors were described.

This paper presents the results of fruit and vegetable comminution experiments carried out with equipment based on mechanical effect. Conclusions are drawn from the results of the present experiments as well as from those published earlier.

1. Materials and methods

1.1. Materials

Quince, plum, apricot and tomato juices were used in the comminution experiments.

The relative particle density of all the juices was taken uniformly as 1.08 $g \cdot cm^{-3}$.

The preparation of the materials and the determination of the relative particle density were performed as described in detail in the earlier paper. Thus the suspensions used contained apart from the dispersed particles only distilled water.

1.2. Methods

1.2.1. Comminutors. Two comminutors based on mechanical effect were used in the experiments. The concentration of the suspensions was adjusted to the concentration required by the construction of the machine; thus the possibility of using suspensions of equal concentration was excluded.

1.2.1.1. MSE homogenizer. — This is a desk-size laboratory equipment. Comminution is performed by stainless steel mixer blades, immersed in the material to be disintegrated and driven by a high capacity electric motor. According to the quantity of the sample, beakers of 3 to 100 ml capacity and mixing blades of appropriate size are interchangeable. The speed is variable between 0 to 14 000 rpm (MEASURING AND SCIENTIFIC EQUIPMENT LTD, 1963). In the course of these experiments 80-ml suspensions of 0.48% were treated in a beaker of 100 ml capacity at maximum speed, for 1, — 2, — 4, — 8-minute periods, respectively.

1.2.1.2. Fryma mill with corundum discs. — Specially manufactured for fine comminution of foodstuffs. The milling process is performed between a disc rotating at about 3 000 rpm and a stationary disc. Different grades of fineness may be obtained by adjusting the grinding gap and by choosing corundum discs of suitable hardness and coarseness (FRYMA, Prospectus).

In these experiments the apparatus, type R, of the Research Institute of the Canning and Paprika Industry, Budapest, was used. The apparatus has a speed of 2 860 rpm. Discs of various coarseness were used, marked as follows: 36P9V85, 46P9V85, 60P9V85 and 80P9V85. Since grain-size is determined by the first two-digit number, hereafter to characterize the discs only these numbers will be used. The higher the number the finer is the granulation.

In every experiment two discs of different coarseness were used for comminution in the following combinations: 46/36, 60/46, 80/60.

Since step by step adjustment of the grinding gap was not possible it could be characterized only by the revolutions of the control knob. In these experiments grinding gaps corresponding to 0.5, 1 and 2 revolutions were applied, using the pair of corundum discs: 80/60. When the effect of the coarseness of discs was tested the grinding gap was set at 0.5 revolution.

In experiments with the Fryma mill 0.1% suspensions were used throughout.

The materials tested were quince and apricot fiber suspensions.

1.2.2. Method of dispersoid-analytical tests. The distribution of particle sizes was established by the sedimentation technique elaborated and published in detail by the author (URBÁNYI, 1968).

Measurements were performed at 20 ± 0.1°C, using suspensions of 0.03% dry fibre content. The particle diameters selected as points of measurement were 160, 105, 68, 44, 28, 17, 10, 5 μm.

1.3. Presentation and evaluation of results

Data obtained by dispersoid-analysis were used to construct granulometric curves. In certain cases — for the sake of illustration — the particle size distribution data were calculated for 5 μm fractions and these were also graphically represented.

The *Trask* parameters were used for column diagrams.

The change of the median ($\Delta\ Md$) was calculated as per cent of the median obtained at the preceding lower degree of comminution and from this was derived the median for unit comminuting action ($\Delta\ Md_u$).

In comparing the results obtained at different degrees of comminution they were analysed mathematically by Student's t test, to establish whether or not the granulometric data of suspensions obtained by increased comminution differed significantly.

Again Student's t test was used to identify the degree of comminution at which the granulometric data differ significantly from those of the original system. The minimum treatment causing a significant change at least at the 95% probability level was designated as Z.

The data given represent the average of 5 parallel measurements.

2. Results

2.1. Examination of fibre suspensions homogenized with the MSE comminutor

In testing the MSE homogenizer time periods of 1, 2, 4 and 8 minutes were applied.

2.1.1. Comminution of quince fibre suspension. Granulometric curves obtained from the results show the comminuting effect of the apparatus (Fig. 1).

As illustrated by the parameters in Fig. 2 a rapid reduction of particles takes place during the first minute of treatment ($\Delta\ Md = 40.50\,\%$), while later this effect substantially diminishes. Between 1 and 2 minutes treatment the change of the median amounts to 5.76 % only, and between 2 and 4 minutes it is 6.95 % ($\Delta\ Md_u = 3.47\,\%$). It is of interest to note that with prolonging the time of treatment the value of $\Delta\ Md_u$ increases and between 4- and 8-minute treatments amounts to 4.62 %.

Statistical analysis of the granulometric data has shown a highly significant reduction of particle size — with the exception of the smallest par-

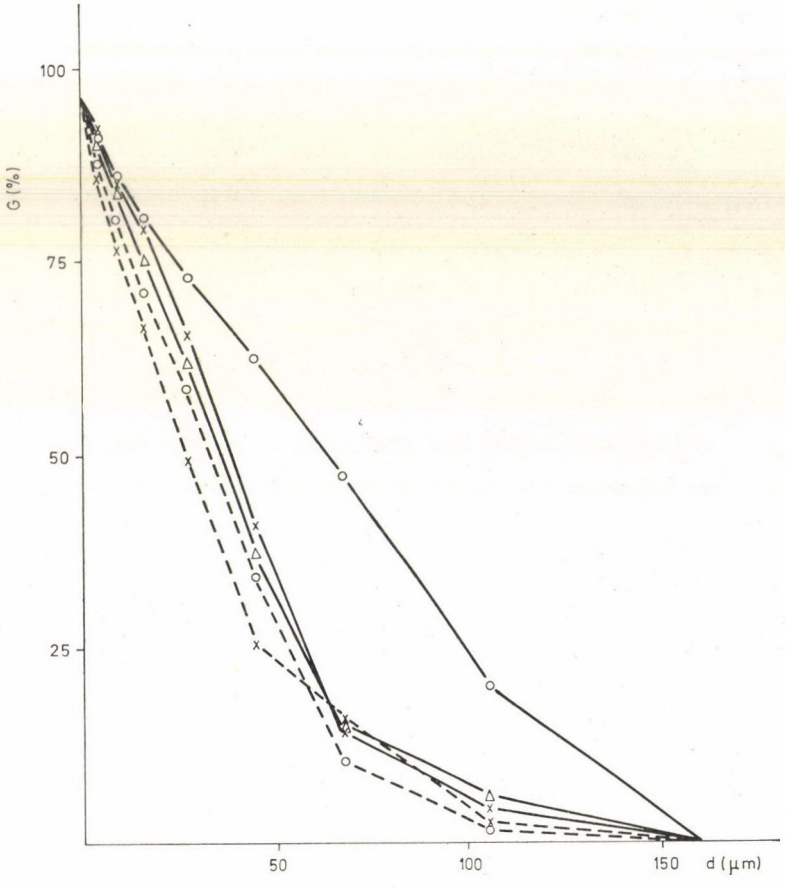

Fig. 1. Granulometric curves of quince fibre suspensions comminuted with the MSE homogenizer. —○— original; —×— comminuted for 1 minute; —△— comminuted for 2 minutes; --○-- comminuted for 4 minutes; --×-- comminuted for 8 minutes

ticles — upon 1 minute of treatment (Table 1). The comparison of suspensions obtained by 1- and 2-minute treatments, respectively, shows an increase in the granulometric data in points corresponding to 105 and 68 μm, however, this

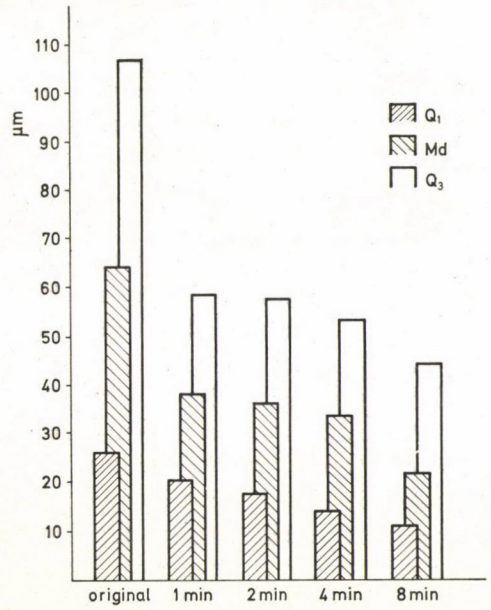

Fig. 2. Parameters of quince fibre suspensions comminuted with the MSE homogenizer

Table 1

Results of the statistical analysis of dispersoid-analytical investigation of quince fibre suspension comminuted with the MSE homogenizer

(P% — level of significance; Z — minimum of treatments required to produce a particle size differing from the initial size distribution at 95% probability level)

d (μm)	Level of significance of the difference between treatments (P%)				Z
	Comminution times (min)				
	0—1	1—2	2—4	4—8	
160	—	—	—	—	—
105	> 99.9	75.0	98.0	< 50.0	1
68	> 99.9	< 50.0	> 99.9	> 99.9	1
44	> 99.9	> 99.9	95.0	> 99.9	1
28	> 99.9	99.0	99.0	> 99.9	1
17	99.0	> 99.9	> 99.9	99.0	1
10	75.0	90.0	> 99.9	> 99.9	2
5	< 50.0	95.0	98.0	98.0	2

is not significant. At all the other points, except at 10 μm, a significant shift toward smaller particles was observed. On comparing the data obtained by 2- and 4-minute treatments significant reductions were observed at every point of measurement. Excepting the two largest particle sizes, similar observations were made on comparing the 4- and 8-minute treatments.

As seen from the results the possibilities provided by the MSE apparatus were not fully exhausted in this series of experiments, presumably the extension of treatment time would have led to a higher degree of dispersion.

A treatment of 2 minutes seems sufficient to obtain a suspension the granulometric data of which differ significantly from those of the original juice in every point of measurement.

2.1.2. Comminution of plum fibre suspension. A substantial reduction of particle sizes was achieved by 1-minute treatment with the MSE apparatus. Extension of treatment time did not result in important changes (Fig. 3).

As shown by statistical analysis (Table 2), the granulometric data of the suspension treated for 1 minute are significantly lower than those of the original, except at point 68 μm. Between treatments for 1 and 2 minutes, data from point 68 μm to 28 μm were increasing. Since, however, treatment for 4 minutes shows again reduction of particles it is evident that these data are due to error. Comparing the suspension treated for 8 minutes with the result of a 4-minute treatment significant difference was not found in any of the measuring points, thus maximum comminution of the given system, obtainable with the MSE apparatus, was achieved.

Table 2

Results of the statistical analysis of dispersoid-analytical investigation of plum fibre suspension comminuted with the MSE homogenizer

(P% — level of significance; Z — minimum of treatments required to produce a particle size differing from the initial distribution at 95% probability level)

d (μm)	Level of significance of the differences between treatments (P%)				z
	Comminution times (min)				
	0—1	1—2	2—4	4—8	
160	—	—	—	—	—
105	95.0	< 50.0	< 50.0	50.0	1
68	50.0	75.0	75.0	75.0	—
44	> 99.9	98.0	90.0	75.0	1
28	> 99.9	99.0	99.0	< 50.0	1
17	> 99.9	50.0	> 99.9	< 50.0	1
10	> 99.9	90.0	95.0	75.0	1
5	> 99.9	95.0	50.0	< 50.0	1

As seen, 1 minute was sufficient to obtain a suspension significantly differing from the original at every point of measurement. At point 68 μm the difference is not significant, however, an 8-minute treatment was not enough either to obtain a significant change in this point.

2.1.3. Comminution of apricot fibre suspension. The results of this series of experiments are similar to those obtained with plum suspension, but a higher degree of dispersion was achieved in the first minute ($\Delta Md = 50.41\,\%$).

Significance tests show (Table 3) that to extend the period of treatment over 4 minutes is not expedient in the case of apricot suspension and MSE apparatus.

Table 3

Results of the statistical analysis of the dispersoid-analytical investigation of apricot fibre suspension comminuted with the MSE homogenizer

(P% — level of significance; Z — minimum of treatments required to produce a particle size differing from the initial size distribution at 95% probability level)

d (μm)	Level of significance of the differences between treatments (P%)				Z
	Comminution times (min)				
	0—1	1—2	2—4	4—8	
160	—	—	—	—	—
105	75.0	< 50.0	75.0	< 50.0	—
68	< 50.0	90.0	50.0	90.0	—
44	> 99.9	< 50.0	75.0	50.0	1
28	> 99.9	< 50.0	95.0	< 50.0	1
17	> 99.9	75.0	98.0	< 50.0	1
10	> 99.9	< 50.0	98.0	< 50.0	1
5	> 99.9	90.0	99.8	50.0	1

In comparing the comminuted suspensions with the original it is seen that 1 minute is sufficient to achieve significant change in the region below 44 μm, but at points corresponding to 105 and 68 μm similar results were not achieved by the increase of treatment time. Since the extension of the treatment at these points brought about changes indicating increase of the particle sizes, this does not seem suitable to achieve the desired result. Thus 1 minute may be considered the time needed to obtain a suspension of significantly reduced particles.

2.1.4. Comminution of tomato fibre suspension. As seen from the results the particles of the initially relatively highly dispersed suspension could not be further comminuted with the MSE homogenizer (Fig. 4).

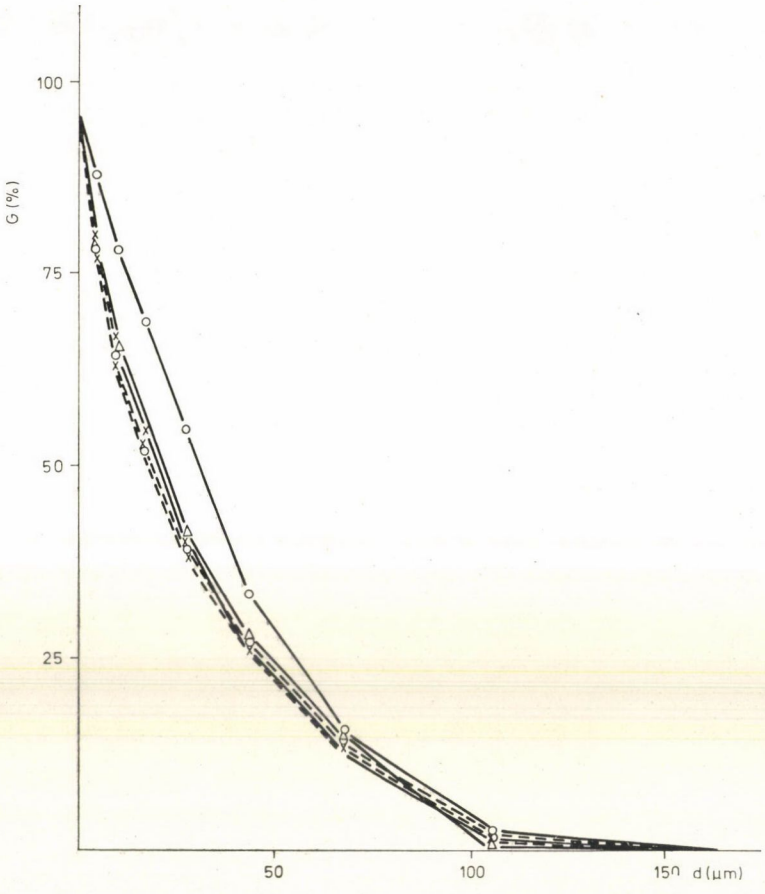

Fig. 3. Granulometric curves of plum fibre suspensions comminuted with the MSE homogenizer. —○— original; —×— comminuted for 1 minute; —△— comminuted for 2 minutes; --○-- comminuted for 4 minutes; --×-- comminuted for 8 minutes

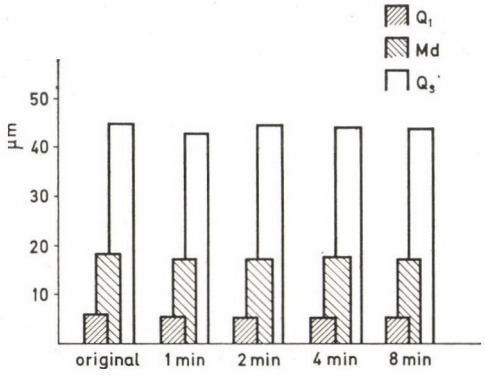

Fig. 4. Parameters of tomato fibre suspensions comminuted with the MSE homogenizer

Significance testing of the results has shown that mathematically valid reduction of particles could not be achieved even by the first treatment. Significant differences were not observed between further treatments, either. Even the 8-minute treatment did not result in a suspension significantly differing from the original.

2.2. Examination of fibre suspensions comminuted with the Fryma mill

Two series of experiments were carried out with this apparatus. In the first series the effect of the coarseness of the corundum discs was studied using in every experiment the same grinding gap of 0.5 revolution. In the second series the effect of various grinding gaps was investigated with the finest pair of corundum discs, marked 80/60.

The test material used in both series was quince and apricot fibre suspension.

2.2.1. Study of fibre suspensions comminuted with discs of various coarseness.

2.2.1.1. Comminution of quince fibre suspension. — Granulometric curves of suspensions obtained by comminution with discs of various coarseness are shown in Fig. 5. The dispersion achieved by all three pairs of corundum discs was higher than that of the original suspension. The highest degree was obtained with the pair of discs marked 60/46, denoting medium coarseness.

The particle size distribution curves (Fig. 6) show that, while the particle size distribution of the original suspension is almost homogeneous in the range between 10 and 105 μm, and the quantity of particles in fractions above 105 μm is also high, as an effect of comminution the amount of fibres in the range above 105 μm is considerably reduced. A maximum was observed in the range between 45 and 65 μm. The highest value, 6.30% per every 5 μm fraction, was achieved with disc pair 60/46. Toward smaller particle sizes the fibre content of the fractions decreases and in the range between 0 and 15 μm the suspensions obtained by all three treatments resemble the original system.

To reveal the correlation between the coarseness of the corundum discs and the granulometric data of the suspensions obtained with them, as well as to establish whether there was a significant difference in comparison to the original suspension, the data were mathematically evaluated (Table 4).

The data show that there is no significant difference, due to difference in coarseness of the corundum discs at any measured point and in any case.

In comparison to the original suspension significant particle size reduction was achieved with all the three disc pairs in the range above 28 μm. At points of measurement below 28 μm no significant difference was found.

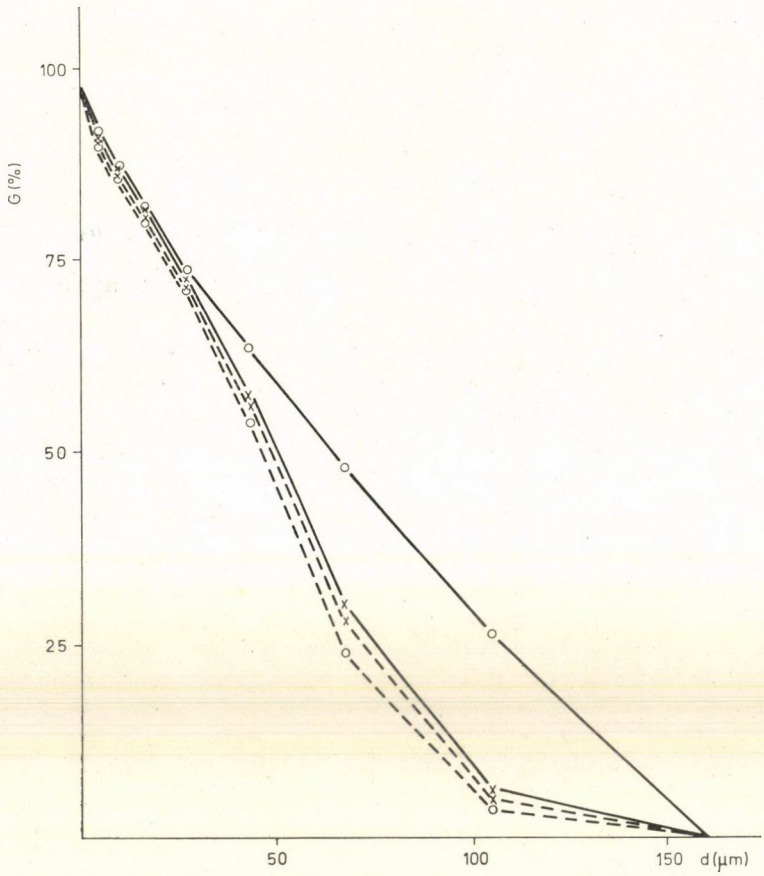

Fig. 5. Granulometric curves of quince fibre suspensions comminuted with the Fryma mill using disc pairs of varied coarseness. —○— original; —×— disc pair marked 46/36; --○-- disc pair marked 60/46; -·×·- disc pair marked 80/60

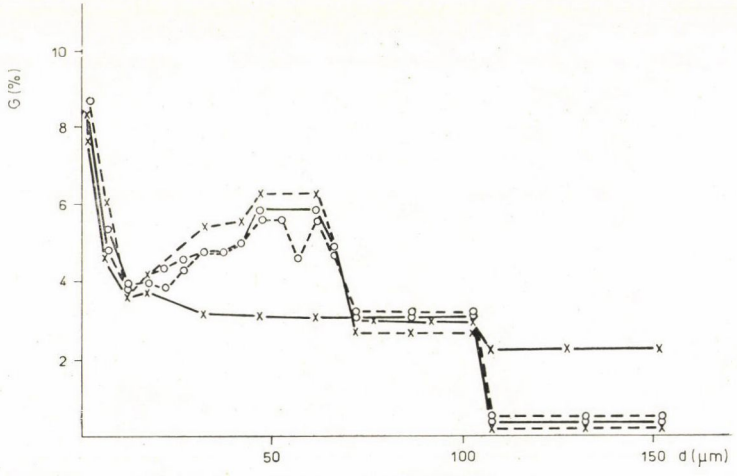

Fig. 6. Particle size distribution curves of quince fibre suspensions comminuted with the Fryma mill using discs of varied coarseness. —×— original; —○— disc pair marked 46/36; --×-- disc pair marked 60/46; --○-- disc pair marked 80/60

Table 4

Results of the statistical analysis of dispersoid-analytical investigations of quince fibre suspensions comminuted with the Fryma mill using discs of various coarseness at a grinding gap of 0.5 revolution

d (μm)	Significance level of the differences between treatments (P%)				
	Suspensions treated with disc pairs of varied coarseness				
	Original-46/36	Original-60/46	Original-80/60	46/36—60/46	60/46—80/60
160	—	—	—	—	—
105	> 99.9	> 99.9	> 99.9	95.0	75.0
68	> 99.9	> 99.9	> 99.9	> 99.9	98.0
44	> 99.9	> 99.9	> 99.9	95.0	98.0
28	95.0	99.0	95.0	75.0	0.0
17	50.0	< 50.0	50.0	< 50.0	< 50.0
10	50.0	75.0	75.0	< 50.0	< 50.0
5	< 50.0	—	—	75.0	75.0

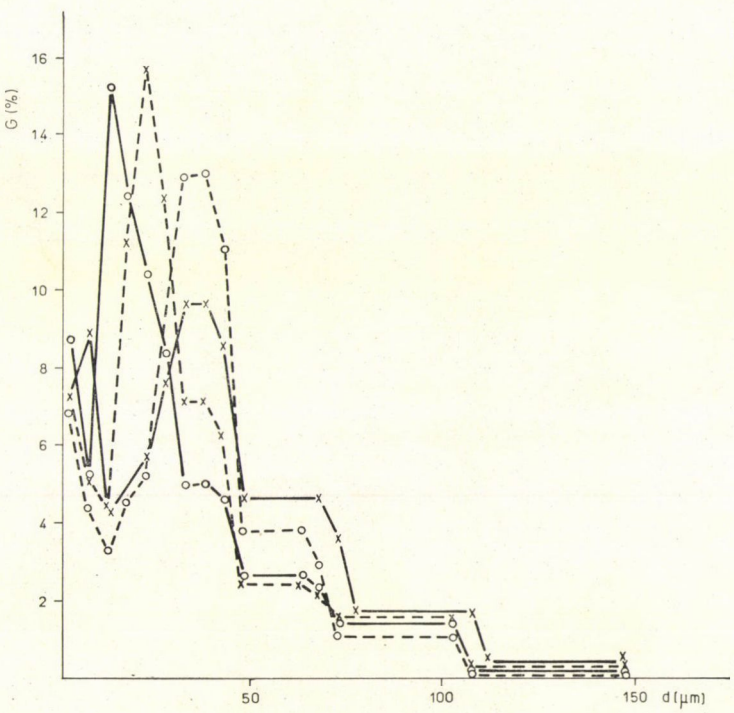

Fig. 7. Particle size distribution curves of apricot fibre suspensions comminuted with the Fryma mill using disc pairs of varied coarseness. —×— original; —○— disc pair marked 46/36; – – × – – disc pair marked 60/46; – – ○ – – disc pair marked 80/60

2.2.1.2. Comminution of apricot fibre suspension. — The results obtained with this test material were more unambiguous than those with quince. The particle size distribution curves (Fig. 7) show that the maximum of the curves is higher if finer discs are used and a shift toward smaller particle sizes is noticeable. The maximum of the original system is in the range of 30—40 μm and has a value of 10.60% per 5 μm fractions. The maximum of the suspension obtained with disc pair 46/36 lays in the same range, but its value is 14.00%. With disc pair 60/46 the maximum is at 20—25 μm (15.73%), with disc pair 80/60 it is at 10—15 μm (15.20%).

Significance tests show (Table 5) more finely granulated disc pairs to produce a higher comminuting effect but the differences in the majority of the measuring points are not significant.

Table 5

Results of the statistical analysis of dispersoid-analytical investigations of apricot fibre suspensions comminuted with the Fryma mill using discs of various coarseness at a distance of 0.5 revolution

d (μm)	Significance level of the differences between treatments (P%)				
	Suspensions treated with disc pairs of varied coarseness				
	Original-46/36	Original-60/46	Original-80/60	46/36—60/46	60/46—80/60
160	—	—	—	—	—
105	< 50.0	75.0	—	< 50.0	95.0
68	95.0	95.0	< 50.0	75.0	90.0
44	99.0	98.0	99.0	90.0	50.0
28	—	> 99.9	> 99.9	> 99.9	98.0
17	—	95.0	> 99.9	98.0	> 99.9
10	—	75.0	> 99.9	75.0	75.0
5	—	50.0	98.0	50.0	90.0

When the granulometric data obtained were compared to those of the original system, it became evident that comminution produced with disc pair 46/36 was not satisfactory, since the comminuting effect was significant only in measuring points corresponding to 68 and 44 μm. With particle sizes below this level the increase of granulometric data was observed.

Using the disc pair 60/46 significant differences were observed between points corresponding to 68 μm and 17 μm, with the disc pair of finest granulation, mark 80/60, the comminuting effect was significant at every point below 44 μm.

2.2.2. Examination of suspensions obtained with various grinding gaps. In the second series of experiments carried out with the Fryma mill the degree of dispersion was studied as a function of the grinding gap. In these tests the disc pair, marked 80/60, was used with grinding gaps corresponding to 0.5, 1 and 2 revolutions of the revolving disc.

2.2.2.1. Comminution of the quince fibre suspension. — As seen in Fig. 8, the degree of dispersion increases only slightly with decreasing grinding gaps.

Statistical analysis of the granulometric data of suspensions obtained with various grinding gaps (Table 6) have shown significant differences only at higher points of measurement.

On comparing the granulometric data of these suspensions to those of the original system it is seen that applying a grinding gap corresponding

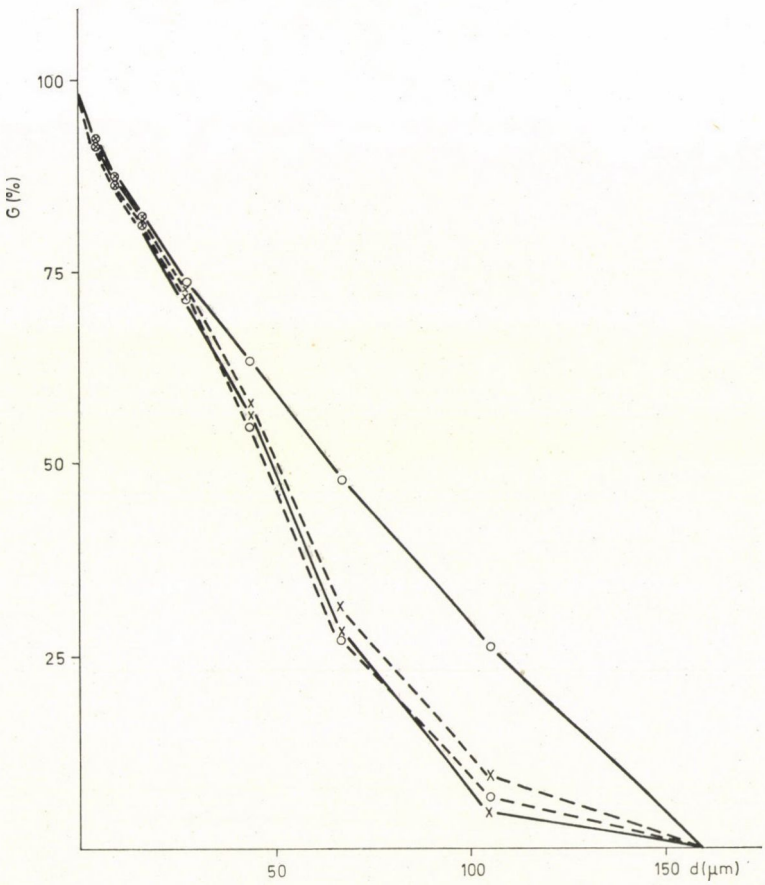

Fig. 8. Granulometric curves of quince fibre suspensions comminuted with the Fryma mill using disc pair 80/60 at various grinding gaps. —○— original; —×— grinding gap set at 0.5 revolution; – – ○ – – grinding gap set at 1 revolution; – – × – – grinding gap set at 2 revolutions

Table 6

Results of the statistical analysis of dispersoid-analytical investigations of quince fibre suspensions comminuted with the Fryma mill using disc pair, marked 80/60 at various grinding gaps

d (μm)	Significance level of the differences between treatments (P%)				
	Suspensions obtained with grinding gaps adjusted by turning of the control knob				
	Original *vs.* 2 revolutions	Original *vs.* 1 revolution	Original *vs.* 0.5 revolution	2 *vs.* 1 revolution	1 *vs.* 0.5 revolution
160	—	—	—	—	—
105	> 99.9	+	+	95.0	98.0
68	> 99.9	+	+	90.0	< 50.0
44	> 99.9	+	+	75.0	50.0
28	< 50.0	98.0	+	50.0	< 50.0
17	—	< 50.0	50.0	< 50.0	< 50.0
10	—	—	75.0	< 50.0	< 50.0
5	—	—	—	< 50.0	< 50.0

+ Since differences at wider grinding gaps were significant it was not necessary to compute these points.

Table 7

Results of the statistical analysis of dispersoid-analytical investigations of apricot fibre suspensions comminuted with the Fryma mill using disc pair marked 80/60, at various grinding gaps

d (μm)	Significance level of the differences between treatments (P%)				
	Suspensions obtained with grinding gaps adjusted by revolutions of the control knob				
	Original *vs.* 2 revolutions	Original *vs.* 1 revolution	Original *vs.* 0.5 revolution	2 *vs.* 1 revolution	1 *vs.* 0.5 revolution
160	—	—	—	—	—
105	—	< 50.0	90.0	75.0	90.0
68	95.0	+	+	95.0	50.0
44	90.0	98.0	+	99.0	50.0
28	—	> 99.9	+	> 99.9	95.0
17	—	> 99.9	+	99.8	99.0
10	—	99.0	+	99.8	50.0
5	—	98.0	+	> 99.9	75.0

to two revolutions, significant changes are observed above 44 μm. Using a grinding gap obtained by one revolution the difference at 28 μm becomes significant. Presumably these grinding gaps correspond to the respective points of measurement.

2.2.2.2. Comminution of apricot fibre suspension. — As seen in Fig. 9, only smaller grinding gaps resulted in a shift toward higher degrees of dispersion.

Statistical analysis of the results shows (Table 7) the grinding gap corresponding to 1 revolution to produce a higher degree of dispersion than that corresponding to 2 revolutions. On further reducing the grinding gap the differences are not significant.

In comparison to the original suspension the system obtained by comminution at a grinding gap corresponding to 1 revolution suffered significant change at all measuring points except at 105 μm. Significant change at this point could not be achieved even by further reduction of the grinding gap.

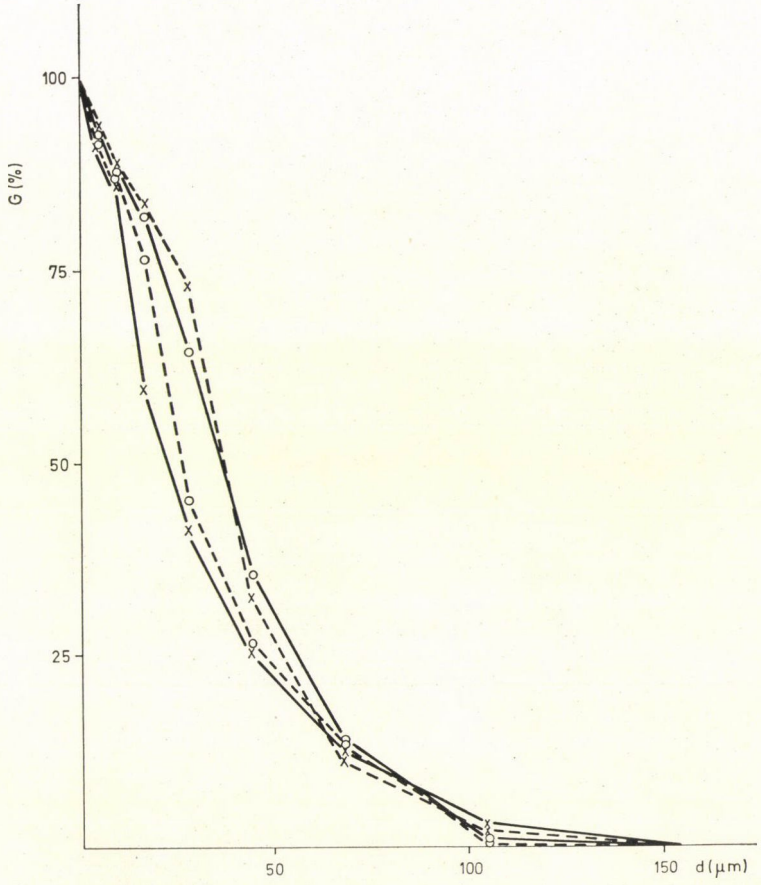

Fig. 9. Granulometric curves of apricot fibre suspensions comminuted with the Fryma mill using disc pair 80/60 at various grinding gaps. —○— original; —×— grinding gap set at 0.5 revolution; --○-- grinding gap set at 1 revolution; --×-- grinding gap set at 2 revolutions

3. Conclusions

3.1. Experiments carried out with the different comminutors

3.1.1. The MSE homogenizer. The experiments with four different test materials have shown that in each case a suspension is obtainable the further dispersion of which with this apparatus is not possible. Of the four test materials used the quince suspension was the only one where even after 8 minutes of treatment significant changes were observed.

With the other three test materials the systems, further dispersion of which was not possible, possessed the following parameters:

	Q_1	Md	Q_3
	μm		
Plums, treated for 8 minutes	6.2	18.7	45.9
Apricots, treated for 4 minutes	5.2	17.2	44.2
Tomatoes, treated for 8 minutes	5.2	17.0	43.5

It is of interest to note that this state was approximated with all three materials after a 1-minute treatment, showing that disintegration takes place mainly during the first part of treatment.

3.1.2. The Fryma mill. Two series of experiments carried out with corundum discs of different coarseness at various grinding gaps have shown the use of more finely granulated discs at small grinding gaps more expedient for the homogenization of plant fibre suspensions. For the selection of the grinding gap the original degree of dispersion of the material has to be taken into account.

3.2. Comparison of the comminutors tested

To characterize the maximum dispersion effect achieved with the different comminutors the values C_{25}, C_{50} and C_{75} were defined as follows:

C_{25} quotient of the lower quartiles of the untreated (original) and the most completely comminuted systems;

C_{50} quotient of the medians for the untreated and the most completely comminuted systems;

C_{75} quotient of the upper quartiles for the untreated and the most completely comminuted systems.

High C-values show high dispersing effect, $C = 1$ means that no comminution took place, that is, the agglomeration equilibrium has set in.

C-values calculated on the basis of the experiments carried out with the two comminutors described above and with the two described in the earlier paper (URBÁNYI, 1972) are illustrated, as functions of the corresponding parameters of the original system, in Figs. 10, 11 and 12.

Since only 2 test materials were tested with the Fryma mill the respective C-values were designated by triangles in the figures.

The peaks in the diagrams of C_{25} values (Fig. 10) are due to the circumstance that the points corresponding to $Q_1 = 26$ μm were obtained with the

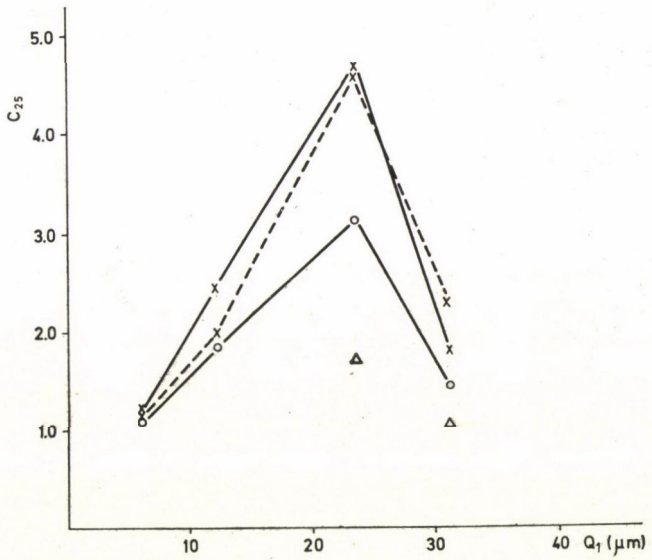

Fig. 10. C_{25}-values changing as a function of the lower quartile (Q_1). × - - - × MSE homogenizer; ×—× Minisonic apparatus; ○—○ MSE ultrasonic apparatus; △ Fryma mill

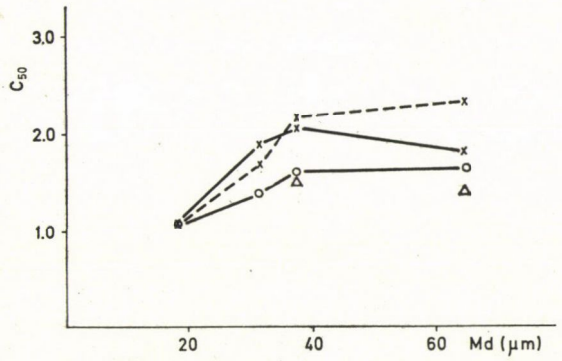

Fig. 11. C_{50}-values changing as a function of the median (Md). × - - - × MSE homogenizer; ×—× Minisonic apparatus; ○—○ MSE ultrasonic apparatus; △ Fryma mill

quince suspension. Since maximum dispersion could not be achieved with the quince suspension, the corresponding C_{25} values were lower than with the other test materials. The anomaly shown by the Minisonic ultrasonic apparatus in Fig. 11 is also due to the same cause. Thus, when drawing the final conclusions, these points were not taken into consideration. The graphs of the C_{75} values (Fig. 12) are of linear character almost to the end. Disregarding points belonging to quince suspensions the rest of the graphs is nearly linear (Figs. 10, 11), too.

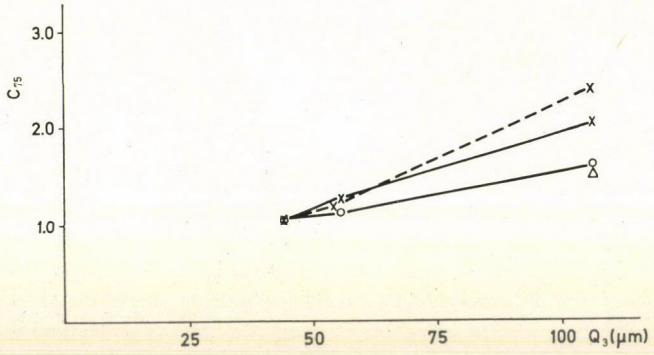

Fig. 12. C_{75}-values changing as a function of the upper quartile (Q_3). × – – – × MSE homogenizer; ×—× Minisonic apparatus; ○—○ MSE ultrasonic apparatus; △ Fryma mil

As can be seen in the figures, the highest degree of dispersion was achieved with the MSE rotary blade homogenizer and the Minisonic ultrasonic apparatus. Both are, in this respect, of the same value.

The lowest degree of dispersion was obtained with the Fryma mill. However, it must be taken into account that the grinding gap of the apparatus used could not be accurately set. With an apparatus more precisely controllable the results would have been probably better.

As seen from the figures, with reduced particle sizes $C \rightarrow 1$, and the differences between the comminuting effect of the different comminutors diminish. With a lower quartile of 5.9 μm the C_{25} values were between 1.11 and 1.20, at a median of 18.3 μm the C_{50} values were between 1.08 and 1.11 and at the upper quartile of 44.8 μm C_{75} values were between 1.04 and 1.05. This shows that in suspensions having these parameters agglomeration is extensive and the system approximates agglomeration equilibrium, therefore further substantial disintegration is not achievable.

In order to be able to determine the parameters of fruit and vegetable fibre suspensions in the state of agglomeration equilibrium, the equations belonging to the linear phase of the disintegration graphs were established. The equations for the different comminutors are as follows:

Minisonic ultrasonic apparatus:

$$C_{25} - 0.1971\,Q_1 - 0.0546 = 0$$
$$C_{50} - 0.0527\,Md - 0.1669 = 0$$
$$C_{75} - 0.0147\,Q_3 - 0.4235 = 0$$

MSE ultrasonic apparatus:

$$C_{25} - 0.1144\,Q_1 - 0.4450 = 0$$
$$C_{50} - 0.0286\,Md - 0.5405 = 0$$
$$C_{75} - 0.0088\,Q_3 - 0.6406 = 0$$

MSE homogenizer:

$$C_{25} - 0.2000\,Q_1 - 0.1843 = 0$$
$$C_{50} - 0.0555\,Md - 0.0350 = 0$$
$$C_{75} - 0.0220\,Q_3 - 0.0285 = 0$$

If in a system under comminution the state of agglomeration equilibrium sets in, then $C_{25} = 1$, $C_{50} = 1$ and $C_{75} = 1$. Solving the above equations for this case the parameters of the system in agglomeration equilibrium are obtained. Calculations led to the following results:

	Q_1	Md	Q_3
	μm		
Minisonic ultrasonic apparatus	4.80	15.80	39.21
MSE ultrasonic instrument	4.85	16.06	40.84
MSE homogenizer	5.90	17.38	44.16

From the above the conclusion can be drawn that a suspension, characterized by these parameters and consisting of solid plant particles dispersed in distilled water, cannot be further dispersed with comminutors of the above construction under the above experimental conditions.

Finally it has to be mentioned that the above particle sizes are valid only on assumption of a particle density of $1.08\ \mathrm{g \cdot cm^{-3}}$. Since there is no absolute method for the determination of the density of plant fibres present in a liquid medium in swollen state, the above data may require modification later on. However, the correction applies only to the absolute values (even these may be converted to values corresponding to another density with the help of a factor). Relative differences between the individual comminutors and comminuting effects remain unchanged whatever the density value of the system.

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DETERMINATION OF THE SIZE OF MYCELIAL PARTICLES BY SEDIMENTATION AFTER DISINTEGRATION IN A CONTINUOUS BEAD MILL

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Mycelial suspensions of *Aspergillus niger* were pumped through a 5-liter bead mill at different speeds to release the intracellular glucose oxidase enzyme by disintegration of the fungal cells.

Dispersoid analytical examinations were subsequently performed with a sedimentation technique to determine the size of particles of the disintegrated cell walls. Mycelial suspensions pumped through the equipment at the lowest pump speeds (10.8 and 19 $l \cdot h^{-1}$) contained in highest percentual proportion the particles falling into the lowest size category (0—5 μm). In the size category of 0—20 μm the occurrence of mycelial particles disrupted at pump speeds of 10.8 and 19 $l \cdot h^{-1}$, proved to be 58 and 52% resp., while in samples disintegrated at pump speeds of 39 and 49 $l \cdot h^{-1}$, only 39 and 37 per cent resp. of the particles were found in the above size category.

The results obtained with the sedimentation technique correlated well with those of enzyme activity assays. The highest enzyme yield was measured in those samples which contained cell fractions of the lowest size category in highest proportions.

Extraction of a metabolic product localized intracellularly within the microorganism is a frequent task in biotechnology. Extraction is preconditioned by disintegration of the cells and the method and instrument of choice for this purpose have to be found for each individual type of microorganism. Determination of the extent of disintegration, *viz.* of the size of the disintegrated cell particles would be helpful not only for the comparison of the individual techniques, but also for the assessment of the necessary extent of disintegration. The microscopic method proposed by KAMNEVA and co-workers (1961) is complicated and also involves considerable sources of error owing to the poor definability of particle surfaces. URBÁNYI (1968) applied with success a sedimentation method for the determination of size distribution of particulate matters in fruit juices and for the control of the disintegration processes as well. This technique seemed to be suitable also for the determination of particle size of mycelia. Measurements of mycelial cell fractions obtained by disintegration in a bead mill at different pump speeds are reported in this paper.

1. Materials and methods

1.1. Test organism

An *Aspergillus niger* strain (PROSZT, 1963) was used as glucose oxidase producing microorganism.

1.2. Cultivation

The above strain was incubated in 10 liter glass fermenters for 24 hours. Inoculum: 10% suspension of a 24-hour vegetative culture. Composition of medium: sucrose: 5%, $Ca(NO_3)_2 \cdot 4H_2O$: 0.4%, $MgSO_4 \cdot 7H_2O$: 0.012%, KCl: 0.025%, corn steep liquor: 2%. The final volume of the medium was 6 liter. The rate of aeration was 1 vol. per vol. per minute with a speed of agitation of 460 rpm, providing for an oxygen supply of 51 mmole $\cdot l^{-1} \cdot h^{-1}$ (COOPER *et al.*, 1944).

1.3. Preparation of the mycelia for disintegration

Mycelia were obtained from the fermentation broth by filtration through a Nylon cloth (mesh: 60 μm). After the removal of medium residues by thorough washing in water, a thick homogeneous aqueous suspension was prepared from the mycelia and used for disintegration.

1.4. Equipment used for the disintegration

The homogenizer is a continuous bead mill (Typ: PM-5, Draiswerke GmbH, Mannheim-Waldhof), consisting of a stand, a 5-liter jacketed container with the agitator, a cog-wheel pump for moving the material to be homogenized to the container through a non-return valve, and two electric motors that drive the pump and the agitator. The container is filled with 6 kg of glass beads of 3 mm diam. The agitator operates at a speed of 1 800 rpm.

1.5. Description of the sedimentation technique

Mycelial particles were separated by centrifugation from the suspensions disintegrated in the bead mill at different pumping speeds. The centrifugal sediment was adjusted to the original volume with distilled water and centrifuged again. This procedure was repeated three times. The clean, washed mycelia were suspended again in distilled water and the dry matter contents of the suspensions were determined. The individual samples were adjusted to approximately identical dry matter contents (about 0.15%).

Prior to the examinations it was established at which particle diameters the measurements should be made. The largest diameter had to be so assessed that no particles greater than that should be present in the system. The following diameter range was selected: 160, 105, 68, 44, 28, 17, 10, 5 μm.

Defining the above particle diameters by equivalent radii, with the sedimentation heights known, the settling times related to the individual diameters were calculated by means of *Stokes*' law

$$t = \frac{9\,h\,\eta}{2\,r^2 g(d_s - d_f)}\,, \tag{1}$$

where t is the settling time (sec), h the height of the settling layer (cm), η the viscosity of the dispersion medium (Poise), r the radius of the particle (μm), g the gravitational acceleration (cm $\cdot$ s^{-2}), d_f the density of the dispersion medium (g $\cdot$ cm^{-3}) and d_s the density of the dispersed part (g $\cdot$ cm^{-3}).

Of the previously prepared suspensions adjusted to an appropriate density, 90 ml amounts each were applied to 5 sedimentation columns (URBÁNYI, 1968) and after homogenization of the material, sedimentation was started.

10-ml fractions collected at predetermined time intervals were poured into previously weighed jars, evaporated by infrared heating and dried overnight at 105°C. Samples were weighed back on a micro-scale (Sartorius MPR 5) after cooling.

From the measured weights of the fractions the granulometric data were calculated as follows.

The total quantity of material settled at time t (P_t) consists of two components. The radii of the particles fully sedimented at time t can be characterized according to *Stokes*' law by the formula (BUZÁGH, 1954):

$$r \geq \sqrt{\frac{h}{kt}}\,, \tag{2}$$

where

$$k = \frac{2}{9} \cdot \frac{d_s - d_f}{n} \cdot g \tag{3}$$

the symbols being the same as in formula (1) and the radii of those particles which had not fully sedimented at time t *viz.* still being in the process of sedimentation (P_{II}) are

$$r \leq \sqrt{\frac{h}{kt}}\,. \tag{4}$$

For dispersoid analysis, the quantity of the fully sedimented particles is needed which, according to the above, is

$$P_I = P_t - P_{II} \,, \tag{5}$$

where

$$P_{II} = t \frac{dG}{dt} \,. \tag{6}$$

Connecting the points of the sedimentation rate curve by straight lines, equation (6) can be formulated as follows

$$P_{II} = t \frac{\Delta G}{\Delta t} \,, \tag{7}$$

where t is the time elapsed between $t = 0$ and the collection of the fraction; ΔG is the weight of the fraction and Δt is the time interval between the collection of two fractions.

The results calculated from the above equations were plotted to obtain granulometric curves. Particle size distribution values were calculated from the granulometric data — at sites between points of measurement by interpolation — and plotted to obtain curves for the distribution of the degree of dispersion. The distribution was calculated for particle width intervals of 5 μm. In the figures the equivalent particle diameters have been designated with d.

1.6. Demonstration of the extent of cell disintegration by an enzymological method

The cell disrupting effect of the bead mill was assessed by determining the activity of the intracellular glucose oxidase enzyme released by disintegration of the cell walls. From 10 ml of a mycelium-containing suspension cell residues were separated by centrifugation at 13 000 rpm in a cooled centrifuge (+5°C) and the supernatant was assayed for glucose oxidase activity.

The degree of cell disruption in the above way was assessed by comparing it to the enzyme yield of mycelia disintegrated by manual grinding with quartz sand for control.

1.7. Determination of the activity of glucose oxidase

Activity of the glucose oxidase enzyme was measured by the method of UNDERKOFLER (1958) as modified by PROSZT (1963) and expressed in Sarrette units (SU).

2. Results

Particle sizes of suspended mycelia of *Aspergillus niger* disintegrated at different speeds in a continuous bead mill were studied by the sedimentation method. The following pump speeds of the bead mill were employed: 2nd stage: 10.8; 3rd stage: 19; 4th stage: 26; 5th stage: 39; 6th stage: 49 $l \cdot h^{-1}$.

Mycelial suspensions disrupted at different pump speeds were compared between themselves. Each sample was sedimented in 5 replicates. The results were plotted out for granulometric curves. From the granulometric data the distribution constants were calculated (at sites between points of the curve by means of interpolation) which were plotted to obtain curves for the distribution of size. Granulometric curves for means of parallel measurements on each sample are shown in Fig. 1, the ordinate showing the percentual distribution and the abscissa the equivalent particle diameters (d) in micrometers.

The granulometric data of all samples are summarized in Table 1.

Table 1

Granulometric data of the mycelia of Aspergillus niger disrupted in a 5-liter bead mill (Typ: PM-5, Draiswerke GmbH, Mannheim-Waldhof) at various pump speeds

The quantity of mycelial particles, larger than the given diameters, as function of the disintegration at different pump speeds. (Means of 5 parallel measurements)

Pump speed ($l \cdot h^{-1}$)	Diameter of disrupted mycelia particles (μm)							
	160	105	68	44	28	17	10	5
	Quantity of mycelial particles (%)							
10.8 (2nd stage)	0.00	0.15	10.85	19.08	27.41	50.05	66.37	79.42
19 (3rd stage)	0.00	0.39	12.80	22.37	33.90	52.32	68.36	83.08
39 (5th stage)	0.00	1.55	14.72	26.80	39.42	56.43	73.07	85.43
49 (6th stage)	0.00	0.52	15.10	27.70	41.76	60.19	76.09	86.98

From data in Fig. 1 and Table 1 it can be established that samples pumped through the mill at lower speeds (2nd and 3rd stages) have shifted towards finer distribution. To facilitate evaluation of certain ranges of size, cumulative distributions were presented each for a given size category.

Figs. 2 and 3 show the granulometric data of the samples as assessed for each range.

As can be seen in Fig. 2 most of the mycelial fractions disintegrated at lower pump speeds belonged to the lower size categories (0—5 and 0—10 μm). At other pump speeds the proportion of particles belonging to the lower size category increased with the decrease of the speed of passage of the mycelial suspension through the mill.

Particles from samples disintegrated at the lowest speed belonged to the size category 0—17 μm in higher proportions (50 %) as compared to those disintegrated at higher speeds. Proportions of the mycelial fractions released at different speeds of the pump were the greater, the faster was the passage of the mycelial suspensions through the mill (Figs. 2, 3).

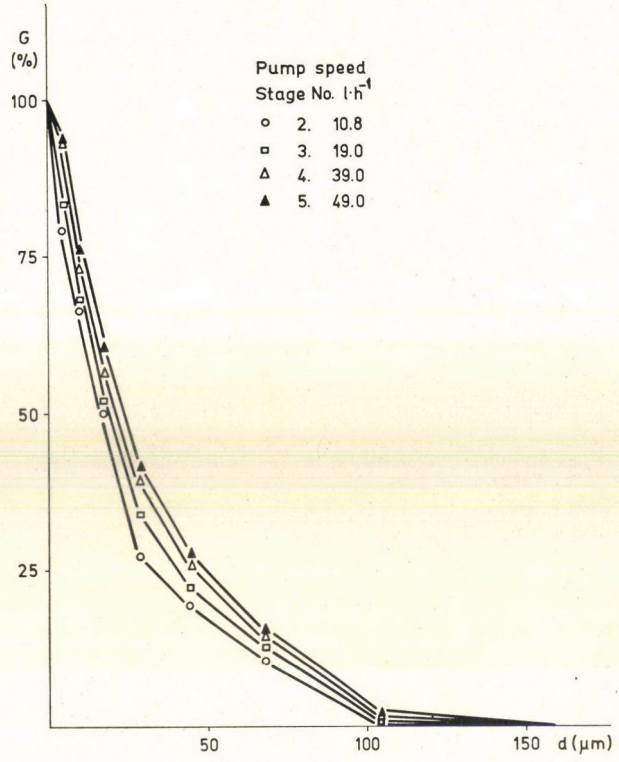

Fig. 1. Granulometric curves of parallel measurements by sedimentation technique of mycelial suspensions from *Aspergillus niger* after disruption in a 5-liter continuous bead mill (Typ: PM-5, Draiswerke GmbH, Mannheim-Waldhof) at various pump speeds (2nd, 3rd, 4th and 5th stages of speed); $d = 2r$ (equivalent diameter of the particles), G = weight of the mycelial particles in per cent

From the granulometric data were calculated the distributions of the degree of dispersity for each sample, related to intervals of 5 μm. Frequency distribution of the various particle sizes were plotted against particle diameters in Fig. 4. The frequency distribution of the various particle sizes as a function of pump speed and of particle diameter was plotted also in a three-dimensional coordinate system (Fig. 5).

Figs. 4 and 5 clearly show that in the lowest size category (0—5 μm) the highest percentual proportions were contained in samples disintegrated at pump speeds of the 2nd and 3rd stages. Samples disintegrated at higher

through-flow speeds showed, in contrast, a distinct shift of particle size toward the higher size categories. The above figures also reveal that the extent of disintegration could be increased by reducing the through-flow speed of the disruption process.

The efficiency of disintegration was checked also by enzymological examination when the output of the endocellular glucose oxidase enzyme

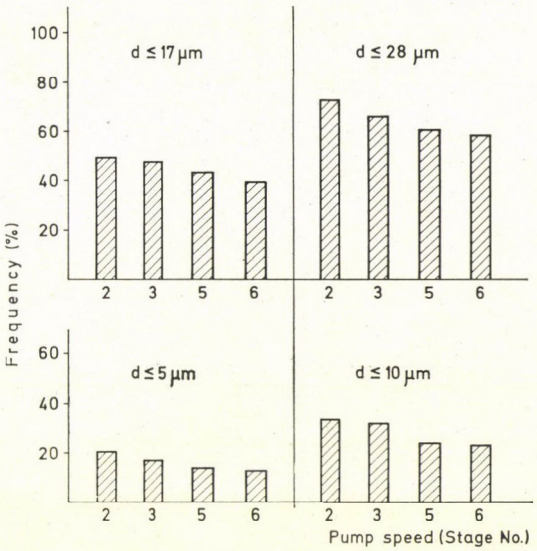

Fig. 2. Cumulative frequency in the 0—28 μm range of particle diameters in mycelial suspensions disintegrated in the continuous bead mill at various pump speeds

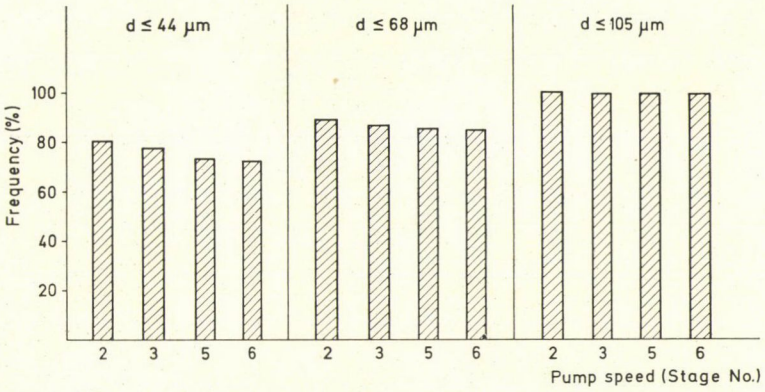

Fig. 3. Cumulative frequency of particle diameters in mycelial suspensions disintegrated in the continuous bead mill at various pump speeds

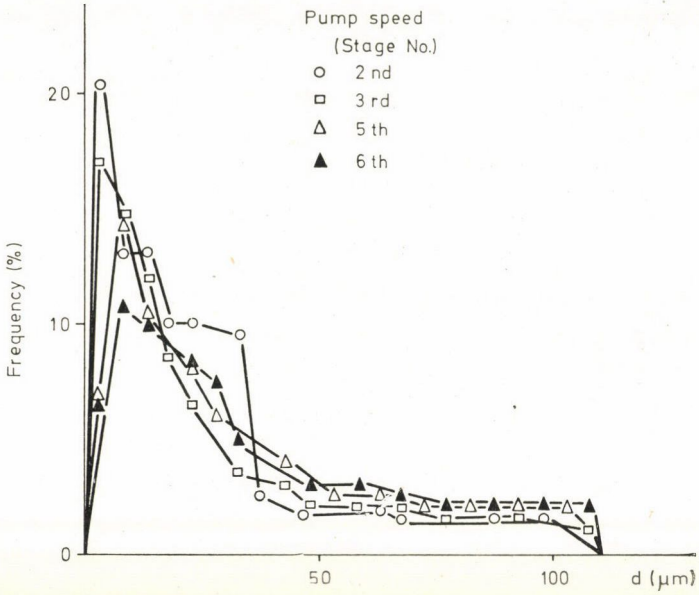

Fig. 4. Particle size distribution of mycelia disrupted at various pump speeds

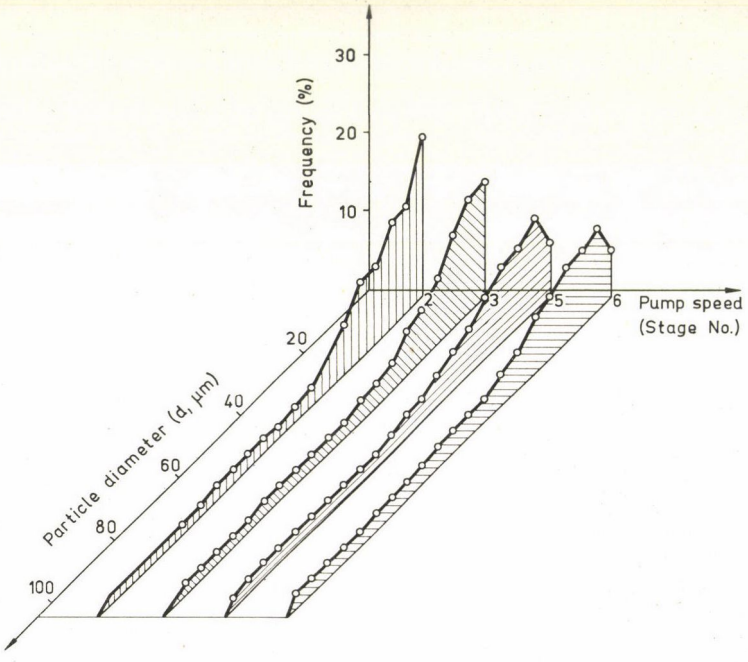

Fig. 5. Frequency distribution of various particle dimensions as a function of pump speed and particle diameter, resp.

was determined. Fig. 6 shows the results of the enzymological examinations. The glucose oxidase activities measured in 10-ml samples each from mycelial suspensions disintegrated at different pump speeds were plotted as functions of the pump speed.

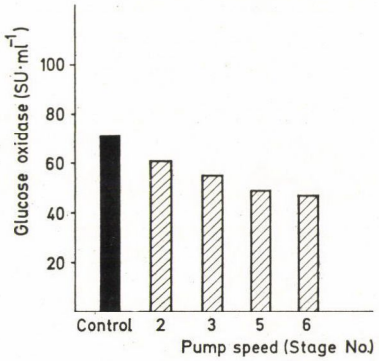

Fig. 6. Total glucose oxidase activity of the supernatants from 10 ml mycelial suspensions disintegrated in the continuous bead mill at various pump speeds

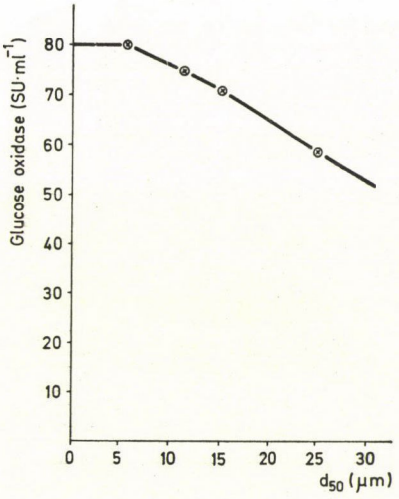

Fig. 7. Glucose oxidase released as a function of medium particle size (d_{50})

As can be seen from the figure, the slower was the passage of the mycelial suspension through the mill, *viz.* the lower pump speed was employed, the higher was the enzyme activity, released from the cell, in identical volumes of the test material.

Data obtained by the sedimentation method in the above experiments were compared with those of enzyme assays performed to assess the efficiency of disintegration from the degree of release of the intracellular glucose oxidase enzyme. In Fig. 7 the total enzyme activity released from the mycelial cells

(enzyme yield) was plotted against mycelial particle size (diameters occurring with a frequency of 50%, d_{50}).

Obviously, loss of enzyme activity was considerably lower in well disintegrated suspensions with predominantly small particle size than in the suspensions with predominantly larger particle size.

3. Conclusions

Procedures to extract intracellular glucose oxidase enzyme from fungal cells were given in previous publications (ZETELAKI 1967, 1969), when the method of choice providing optimal cell disintegration proved to be the bead mill. Optimal enzyme yield *via* disruption of the cell wall could be controlled with the speed of passage of the material through the mill (Fig. 6). Greater release o enzyme occurred with lower through-flow speed, *i.e.* with higher degree of disintegration.

The sedimentation technique was employed to precisely assess the extent of influence of the passage speed of the mycelial suspension through the mill on the size of cell wall particles produced by disintegration. Suspensions passed through the mill at lower pump speeds showed greater proportions of particles in the lowest size category as compared to those passed at higher pump speeds (Figs. 2, 3). The data of the sedimentation studies are in good agreement with URBÁNYI's (1968) finding that repeated comminutions result in a shift of particle size toward the lowest size categories.

Sedimentation tests performed after disruption of mycelial suspensions in bead mill at various pump speeds showed the lowest particle size category, 0—5 μm, to be most frequent in the samples passed at the lowest pump speed. With the increase of the speed of the pump the frequency of particle diameters of 0—5 μm tended to be reduced.

In samples disintegrated at pump speeds of 10.8 and 19 $l \cdot h^{-1}$ (2nd and 3rd stage) the frequency of particles of the 0—5 μm size category was 20.5 and 17%, respectively.

Results obtained by the sedimentation technique and by enzymic assay were in agreement. The greatest amount of intracellular enzyme was obtained from mycelial suspensions in which particles of the lowest size category showed highest frequencies.

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STORAGE EXPERIMENTS WITH THE RAW MATERIAL OF SEASONING PAPRIKA, WITH PARTICULAR REFERENCE TO THE RED PIGMENT COMPONENTS

M. HARKAY-VINKLER

(Received January 6, 1973)

The formation of the colour components of paprika, a measure of its value, was followed during storage of the paprika fruit.

The formation of the pigment content was found to fall into three phases: the initial increasing phase was followed by a stationary phase. During the third phase a substantial reduction was observed.

The maximum of pigment formation was found to occur between the 25th and 39th days of storage.

The major part of the pigment components is formed by capsanthin as established by both the method of BENEDEK (1952) and the thin-layer chromatographic method of VINKLER and KISZEL-RICHTER (1972). The absorption curves characteristic of the pigments are decisively affected by this component.

The market value of ground paprika is determined by its colour and colouring capacity. This necessitates the knowledge of the conditions of maximum pigment formation.

According to the investigations of CHOLNOKY and co-workers (1958), during the first stage of paprika processing, the drying of the harvested paprika fruit, two definite periods may be distinguished. In the first period the paprika pericarp is green, due to its chlorophyll content. The characteristic red colour of seasoning paprika appears in the second period. In the initial phase of the second period a brownish colour becomes apparent which is due to the simultaneous presence of green chlorophyll and red carotenoids. This initial phase lasts only a few days, afterwards the carotenoids prevail.

BENEDEK (1958, 1959) was the first to study in detail the changes occurring during the period following harvest. He has shown, with the help of his method, that during the so-called "after-ripening" period, in the initial phase the pigment content rapidly increases, this is followed by a phase of slower pigment formation and after 4—6 weeks a maximum is reached. Simultaneously with this process the sugar content substantially diminishes.

The details of the "after-ripening" process were studied in a number of papers (BENEDEK *et al.*, 1961, 1964; KARDOS, 1954, 1960; FARKAS, 1961, 1962; PALOTÁS & KONECSNI, 1959; PALOTÁS & MIHALIK, 1963). In these studies the importance of the "after-ripening" process was proven and the differences between various storage methods were numerically established and the effect of advantageous and detrimental changes clarified.

These studies investigated the effects of the process under the conditions of the conventional way of "after-ripening", that is the paprika being stringed and stored in this form. However, due to the significant increase in production the conventional method became unsatisfactory. Thus a new storage method, the so-called "net-sack" method was worked out and will be described further on. The aim of the present study was to elucidate the formation of the red pigments under the new storage conditions.

1. Materials and methods

1.1. Materials

Paprika variety E 15, from the Kalocsa district, first-harvest, mild, selected at the place of cultivation, stored in the new way on about the 10th day after picking, was used as test material.

In three parallel closed cycle experiments 100 kg paprika each was stored.

Method of storage: the freshly picked paprika was placed in sacks prepared from cotton netting of 1 cm^2 mesh, 95 cm long, having a diameter of 18 cm. About 6 kg bell pepper were placed in each sack and the sacks were hung up in a well aired shed and stored under roof till the formation of maximum pigment content.

The experiments were carried out on the premises of the "Gyümölcs- és Főzelékkonzervgyár" (Canned fruit and vegetable factory, Budapest) between October and December 1970. The climatic conditions during storage were recorded.

Month	Average temperature °C	Relative humidity%
October, 1970	9.8	76—80
November	7.0	82—86
December	1.0	83—89

Testing of the samples was carried out between October, 1970 and January, 1971.

1.2. Methods

The pigment content of the dried paprika pericarp was analysed by BENEDEK's method (1952) and the method developed by the author (VINKLER & KISZEL-RICHTER, 1972).

The average sample was obtained by sampling every sack and evening up the sample thus obtained including damaged and deteriorated fruits. The results given here are the averages of three series of experiments.

2. Results

To follow up the changes occurring during storage several parameters affecting the technological and economic aspects were investigated. The present report wishes to evaluate the most important chemical process, the development of the red pigment, the measure of the value of ground paprika, only.

According to the results of these experiments three periods may be distingished during "after-ripening".

Fig. 1 illustrates the formation of the pigment, using the two methods mentioned above. As seen, the pigment content substantially increases till the 18th day of storage, between the 10th and 39th days maintains a relatively constant level, and after the 46th day the pigment formation is slowing down.

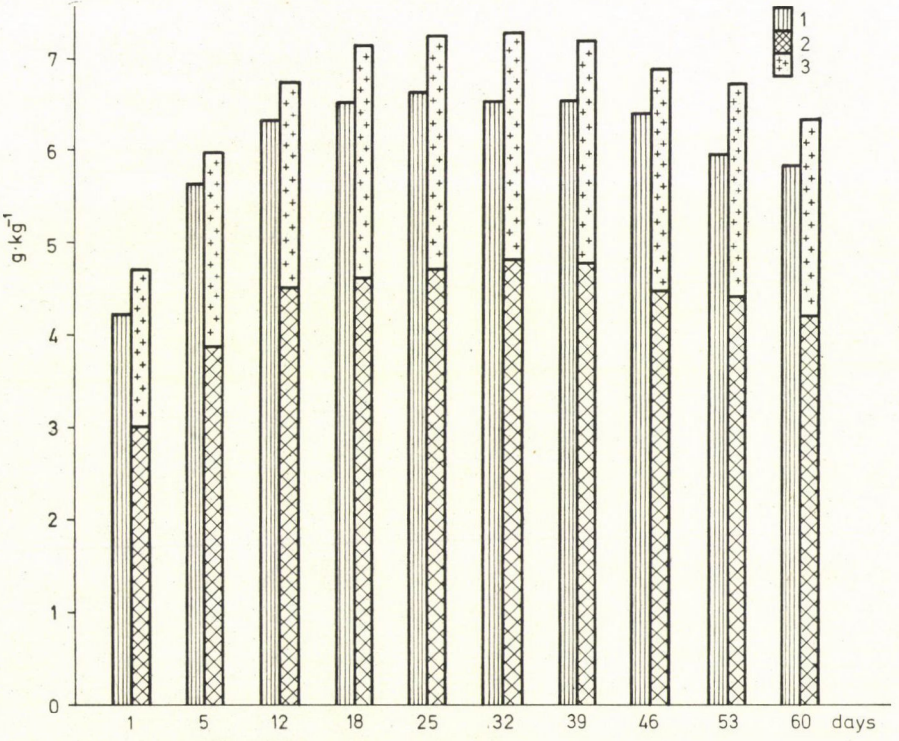

Fig. 1. Pigment content as a function of storage time: 1 — using *Benedek*'s method; 2 — thin-layer chromatography of the red components; 3 — thin-layer chromatography of the yellow components

The same course of formation is shown by all red and all yellow colour components.

A similar course is shown in the quantitative distribution of the colour components (Fig. 2). The quantity of the red component, capsanthin, decisively influencing the pigment content increases substantially up to the 18th—to 25th day of storage. During the same period the quantity of capsorubin increases at a slower rate. Between the 25th and 39th day of storage the level of the red components is maintained and after the 46th day the value of both red components slowly decreases (Table 1).

Table 1

Changes in the pigment components during storage

Storage time, days	Total pigment content according to Benedek (1)	Pigment content (g · kg^{-1}) by thin-layer chromatography							
		Total red (2)	Total yellow (3)	Capsanthin (4)	Capsorubin (5)	Capsanthin + capsorubin	Zeaxanthin + lutein (6)	Beta-carotene + cryptoxanthin (7)	Zeaxanthin + lutein + beta-carotene + cryptoxanthin
1	4.19	3.00	1.72	2.40	0.50	2.90	1.00	0.60	1.60
5	5.60	3.86	2.10	3.06	0.60	3.66	1.36	0.65	2.01
12	6.31	4.48	2.28	3.65	0.67	4.32	1.50	0.70	2.20
18	6.47	4.60	2.50	3.82	0.67	4.49	1.60	0.80	2.40
25	6.60	4.71	2.55	3.82	0.68	4.50	1.60	0.80	2.40
32	6.50	4.80	2.47	4.09	0.63	4.71	1.48	0.85	2.33
39	6.50	4.76	2.40	3.92	0.70	4.62	1.47	0.90	2.37
46	6.41	4.51	2.38	3.81	0.60	4.41	1.41	0.90	2.31
53	5.92	4.42	2.70	3.70	0.60	4.30	1.10	0.85	1.95
60	5.80	4.22	2.15	3.50	0.60	4.10	1.20	0.90	2.10

Percentage distribution of pigment components

				(4)	(5)		(6)	(7)	
1				53.3	11.1		22.2	13.3	
5				53.9	10.5		23.9	11.4	
12				55.9	10.2		23.0	10.7	
18				55.4	9.7		23.2	11.6	
25				55.3	9.8		23.1	11.5	
32				58.0	8.9		21.0	12.0	
39				56.0	10.0		21.0	12.8	
46				56.6	8.9		20.9	13.3	
53				59.2	9.6		17.6	13.6	
60				65.4	9.7		19.3	14.5	

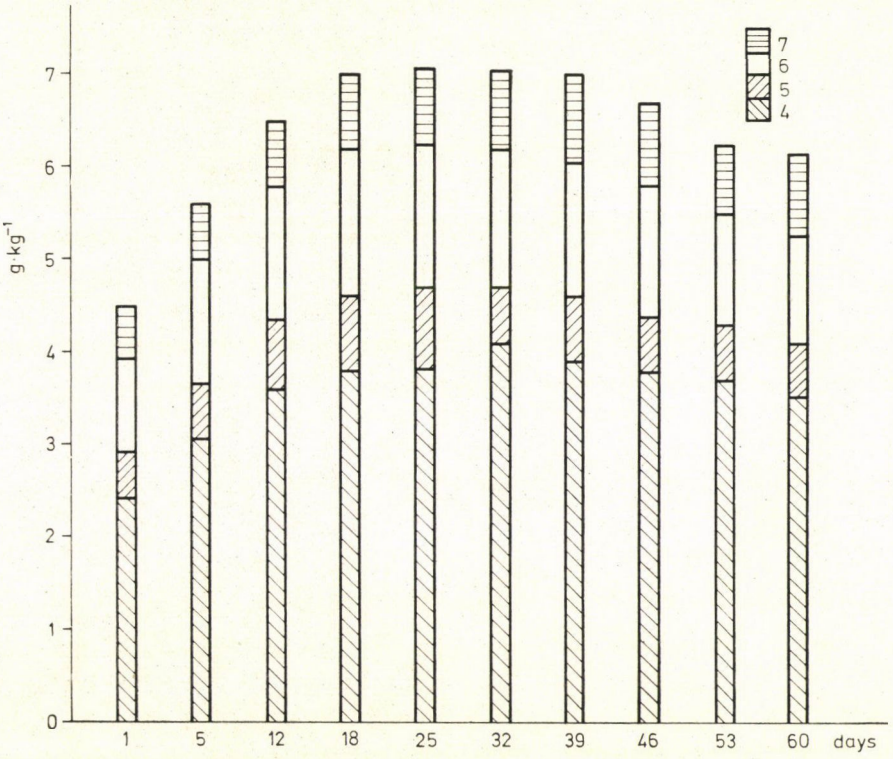

Fig. 2. Quantity of pigment components ($g \cdot kg^{-1}$), as a function of storage time, determined by thin-layer chromatography. 4 — capsanthin; 5 — capsorubin; 6 — zeaxanthin + lutein; 7 — beta-carotene + cryptoxanthin

In Table 2 the standard deviations and the coefficients of variation belonging to the results obtained in the three experimental series are summed up.

The percentage distribution of the colour components (Fig. 3) shows that about 53 to 58% consists of capsanthin, increasing slightly at the beginning, but diminishing after the 39th day of storage. The percentage of capsorubin is highest on the first day of storage and shows a tendency to decrease afterwards. This is due to the fact that the absolute amount of capsanthin increases at a higher rate than that of capsorubin, therefore the percentage value of the former seems to be lower.

To verify the correctness of the results a hypothesis test was applied. It was assumed that the increase, during storage, of the pigment content, related to the absolute solids content, is significant. The t test was applied to the total amount of red pigment, representing the commercial value of paprika, by relating the values measured daily to the first measurement, *i.e.* the original value.

Table 2

Standard deviations and coefficients of variation

Storage time, days	Total pigment content according to Benedek (1)			Total red pigment content (2)			Total yellow pigment content (3)		
	$\bar{x}$	s	v	$\bar{x}$	s	v	$\bar{x}$	s	v
1	4.19	0.126	3.02	3.00	0.099	3.32	1.72	0.063	3.71
5	5.60	0.163	2.92	3.86	0.132	3.42	2.10	0.072	3.47
12	6.31	0.187	2.97	4.48	0.174	3.90	2.28	0.088	3.87
18	6.47	0.206	3.17	4.60	0.137	2.99	2.50	0.117	4.71
25	6.60	0.165	2.51	4.71	0.160	3.41	2.55	0.105	4.12
32	6.50	0.223	3.44	4.80	0.153	3.20	2.47	0.100	4.07
39	6.50	0.232	3.57	4.76	0.189	3.99	2.40	0.108	4.50
46	6.41	0.282	4.42	4.51	0.185	4.11	2.38	0.118	4.97
53	5.92	0.188	3.20	4.42	0.208	4.71	2.70	0.105	3.91
60	5.80	0.191	3.31	4.22	0.168	4.00	2.15	0.086	4.04

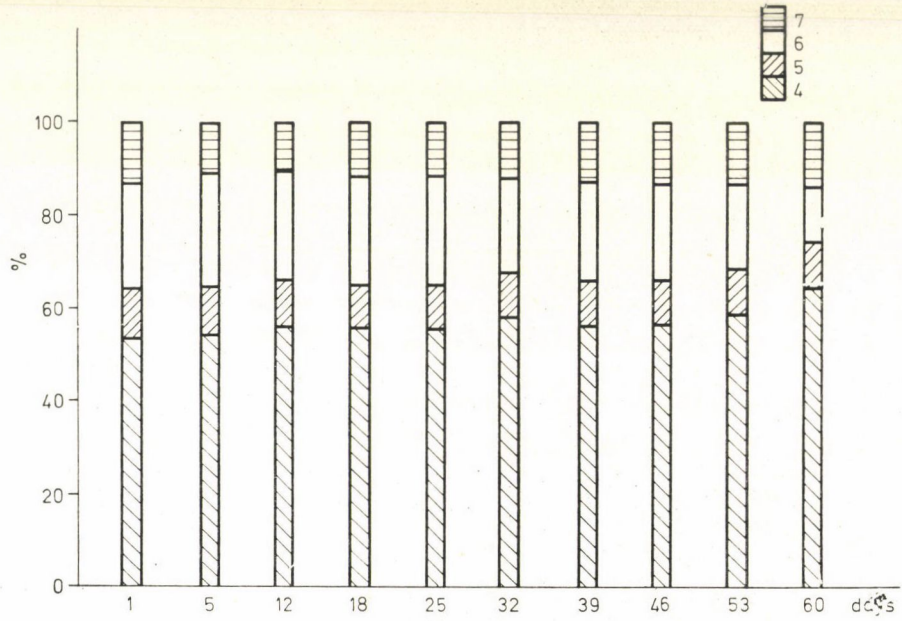

Fig. 3. Percentage distribution of the pigment components as a function of storage time, determined by thin-layer chromatography. 4 — capsanthin; 5 — capsorubin; 6 — zeaxanthin + lutein; 7 — beta-carotene + cryptoxanthin

of the results of the three experimental series

Capsanthin (4)			Capsorubin (5)			Zeaxanthin + lutein (6)			Beta-carotene + cryptoxanthin (7)		
$\bar{x}$	s	v	$\bar{x}$	s	v	$\bar{x}$	s	v	$\bar{x}$	s	v
2.40	0.052	2.18	0.50	0.023	4.58	1.00	0.059	5.90	0.60	0.026	4.42
3.06	0.123	4.04	0.60	0.030	5.00	1.36	0.071	5.28	0.65	0.025	3.90
3.65	0.113	3.11	0.67	0.022	3.32	1.50	0.077	5.14	0.70	0.032	4.71
3.82	0.142	3.72	0.67	0.026	3.97	1.60	0.096	6.02	0.80	0.043	5.36
3.82	0.153	4.01	0.68	0.039	5.81	1.60	0.091	5.72	0.80	0.035	4.40
4.09	0.200	4.90	0.63	0.026	4.17	1.48	0.086	5.82	0.85	0.051	6.10
3.92	0.199	5.10	0.70	0.035	5.00	1.47	0.057	3.90	0.90	0.046	5.10
3.81	0.237	6.24	0.60	0.016	2.72	1.41	0.070	4.99	0.90	0.045	5.00
3.70	0.123	3.34	0.60	0.036	5.14	1.10	0.056	5.10	0.85	0.032	3.77
3.50	0.170	4.87	0.60	0.021	3.55	1.20	0.036	3.00	0.90	0.038	4.27

$\bar{x}$ = mean of three measurements ($g \cdot kg^{-1}$)
s = standard deviation ($g \cdot kg^{-1}$)
v = coefficient of variation (%)

Alternative hypothesis: the red pigment content is increased by the treatment (one sided hypothesis).
Level of significance: $\alpha = 5\%$,
basis of the test statistics: $t = \dfrac{\bar{d}}{\bar{S}} \sqrt{n}$,

$\bar{d}$ = mean of degrees of freedom,
$\bar{S}$ = mean of standard deviations,
DF = 9,
t_{cal} = 7.93.

Comparative table value for t at $DF = 9$, in case of the one sided alter, native hypothesis $\alpha = 5\%$, : $t = 1.83$.

Since the calculated t value is much higher than the value in the table- the pigment contents as measured daily during storage differ to a significant degree from the original value.

The study of the yellow components has shown a rapidly increasing tendency of the zeaxanthin + lutein components, while the composite value of beta-carotene + cryptoxanthin hardly changes and on the whole remains on the same level till the end of the storage period. The composite value of zeaxanthin + lutein diminishes extensively after the 46th day of storage (Fig. 2).

The formation of the pigment content or of the components composing the pigment content is more clearly illustrated if the values, as measured by both methods *(Benedek's* method and thin-layer chromatography) on given days of storage, are characterized by absorption curves.

The values established on certain days of storage are related to a concentration of 1 $g \cdot l^{-1}$ in the Figures which follow.

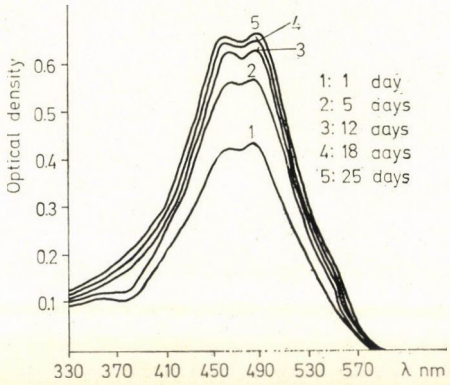

Fig. 4. Absorption curves of the benzene extracts (1 $g \cdot l^{-1}$) of the stored samples obtained by *Benedek*'s method.

Storage time (days)	Optical density at 488 nm
1	0.435
5	0.569
12	0.630
18	0.652
25	0.664

Fig. 4 shows the absorption diagrams of the total pigment content as established by *Benedek*'s method in the benzene extract. On the first day of storage the maximum was observed at 465 and 488 nm. The maximum at 488 characterized the red components, while the peak, at 465 nm the yellow components. The two maxima are not too pronounced on the first day. With increasing pigment content the extinction values of the curves become higher, the peaks are pronounced, however, the wavelength of peaks remains unchanged.

In the methyl alcohol solutions obtained in thin-layer chromatography the red component mainly consists of capsanthin (Fig. 5). Since the quantity of capsorubin in relation to capsanthin is irrelevant the absorption curves

thus obtained are characteristic of capsanthin. All the curves belonging to the red components are similar and correspond to the capsanthin curve.

The yellow components were eluted together with methanol and the absorption curves were plotted versus storage time (Fig. 6). The maximum of every component is at the same wavelength, thus the maximum of the curves resulting from these components is at wavelength 445 nm.

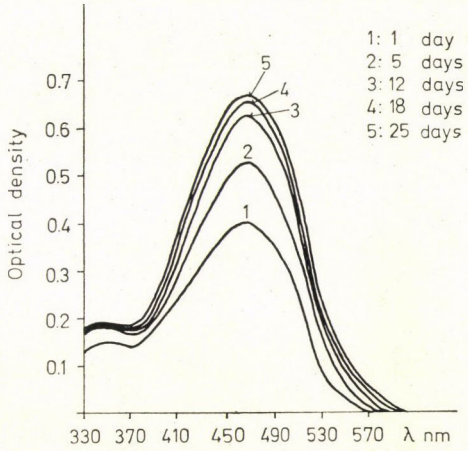

Fig. 5. Absorption curves of the red pigment components of the benzene extract of paprika eluted with methanol (concentration of the methanol solution: 1 g · l^{-1}), layer: Kieselgel G.

Storage time (days)	Optical density at 465 nm
1	0.400
5	0.525
12	0.628
18	0.665
25	0.671

The combined zeaxanthin and lutein content is 40% higher at the beginning of the storage period than the beta-carotene and cryptoxanthin content, thus in the absorption curve of all the yellow components the character of the zeaxanthin and lutein content is predominant.

3. Conclusions

(a) The red components consist in the majority of capsanthin thus the low capsorubin content has no effect on the shape of the absorption curve characteristic of all the red components.

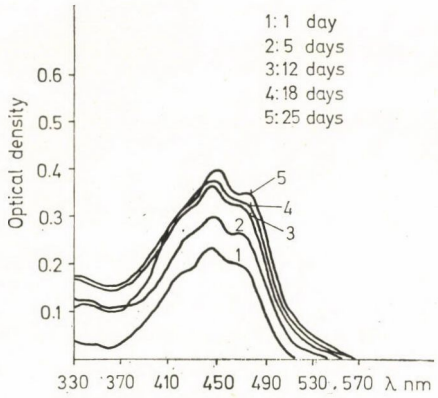

Fig. 6. Absorption curves of the yellow pigment components in the benzene extract o: paprika eluted with methanol (concentration of the methanol solution: 1 g · l^{-1}), layerf Kieselgel G.

Storage time (days)	Optical density at 445 nm
1	0.233
5	0.300
12	0.365
18	0.376
25	0.397

(b) The period of "after-ripening" falls into three phases. The pigment content is formed during the first phase. After the appropriate bound water content is reached in the second phase the enzymes of paprika become inactivated and there is no further pigment formation, a state of equilibrium sets in. After about the 46th day a reduction of the pigment content may be observed. This is highly affected by the conditions of storage, or in other words, by the success, or otherwise, in eliminating the deterioration and discoloration. In the experiments described, 25 to 39 days seemed to be the period most advantageous for after-ripening. During this period maximum pigment content was achieved.

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THE ROLE OF WATER RADICALS IN THERMORESTORATION OF BACTERIAL SPORES

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Fully hydrated bacterial spores exposed to 0.45 Mrad showed a characteristic pattern of survival associated with thermorestoration. When temperature during radiation was controlled at −15° to +120°C, the lowest viable cell counts were at 0°C. Above 0°C radiosurvival gradually increased by 2 to 3 log cycles reaching peak at 75°C (*Bacillus cereus* T heat sensitive spores) and at 95°C (*B. stearothermophilus*, heat resistant spores). Simultaneously high survival was observed in the solidly frozen state at −15°C to −5°C since harmful radicals produced by radiation were trapped in ice.

Radiation modifying effects, *i.e.*, protection by 2*M* ethanol (a scavenger of ·OH radicals) and sensitization by 1*M* sodium nitrate (a scavenger of H· radicals and hydrated electrons), were most pronounced at 0°C where radicals were most abundant. Radiation modifying effects were lowest at 50°C to 70°C where water radicals were competitively annealed by heat and hence not available. The effects of ethanol and nitrate were also low at −5° and −15°C where radicals were trapped in ice. Generally, radiation sensitization by nitrate was much more pronounced with the radiation sensitive spores of *B. stearothermophilus* as compared with the radiation resistant *B. cereus*. On the other hand, radiation protection by ethanol was more pronounced with the radiation resistant *B. cereus*, as opposed to smaller protection of *B. stearothermophilus*. While ethanol protects the spores against radiation at temperatures below 50°C by scavenging ·OH radicals, the effect of ethanol was distinctly reversed at irradiation temperatures above 50°C. The results with ethanol and nitrate confirm the idea that in aqueous systems below 50°C the lethal action is due to oxidizing ·OH radicals known to attack cell DNA. However, the reversal of scavenger actions above 50°C indicates that at those high temperatures lethal effects may also involve the reducing H· and e^-_{aq}, which at lower temperatures appear not to affect spore survival though they are known to attack proteins. In this case, it is proposed that radiation inactivation of spores at temperatures below 50°C is due to DNA damage inflicted by ·OH radicals whereas spore death above 50°C seems to involve protein (enzyme) inactivation due to a combined action of heat plus reducing (H·, e^-_{aq}) as well as oxidizing (·OH) radical species. From the practical point of view it is important that normally radioprotective effects of such substances as ethanol or ground beef are progressively lost when radiation is carried out at temperatures above 50°C.

In an earlier paper, evidence was presented demonstrating the role of cellular determinants, *viz.*, heat resistance, radiation resistance, and the ionic form of spores on the thermorestoration of hydrated spores of *Bacillus cereus* and *B. stearothermophilus* (FRIEDMAN & GRECZ, 1973). By subjecting these organisms to a radiation dose of 0.45 Mrad of ^{60}Co in distilled water at temperature levels between −15° and 120 °C, a characteristic thermorestoration survival pattern was obtained (Fig. 1).

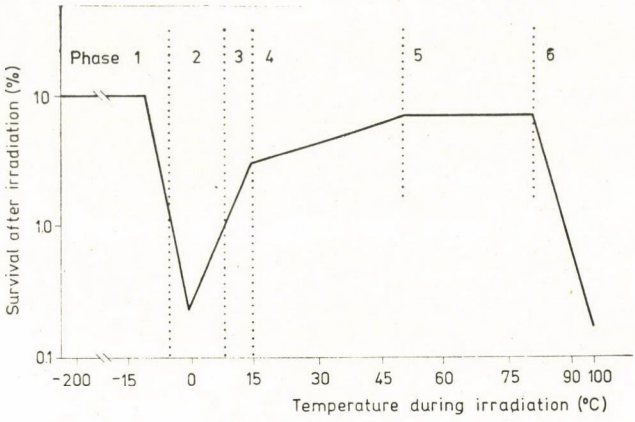

Fig. 1. Thermorestoration survival pattern

This pattern was divided into six phases:

Phase 1. Frozen state below 0 °C where spores exhibit high radiation survival due to the trapping of water radicals by ice.

Phase 2. At 0 °C where spore survival was lowest because of increased water radical mobility after thawing.

Phase 3. Between 0° and 15 °C where a rapid and progressive increase in spore survival was due to increased annealment (thermorestoration) of lethal radicals.

Phase 4. Between 20° and 50 °C where a slow-down of the thermorestoration process occurs.

Phase 5. At temperatures above 50 °C where a complete saturation of the thermorestoration process results in a leveling of the survival curve.

Phase 6. The highly lethal terminal range above 65 °—75 °C for *B. cereus* and above 79 °—95 °C for *B. stearothermophilus.*

From a practical viewpoint, it appears quite attractive to use combinations of heat and radiation for a variety of sterilizing purposes. The possibility of synergistic inactivation by these two energies was a logical expectation of earlier investigators (KAN *et al.*, 1957; KEMPE *et al.*, 1957; KEMPE & GRAIKOSKI, 1959). It soon became evident that the simultaneous administration of heat and radiation showed no simple interactions (GRECZ, 1966; GRECZ *et al.*, 1967). Subsequent investigation indicated that in applying heat plus radiation, complex interactions were elicited at the atomic, molecular and cellular level in the bacterial cell itself (WEBB *et al.*, 1960), as well as radiochemical interactions of radicals in the suspending medium (SUCHANEK *et al.*, 1969; FRIEDMAN & GRECZ, 1973). These factors must all be taken into account

in trying to explain the final result in a particular schedule of treatment involving both heat and radiation.

The highly lethal terminal stage of Phase 6 of the thermorestoration survival pattern is especially conducive to the destruction of spores in simple aqueous solutions (SUCHANEK *et al.*, 1969; FRIEDMAN & GRECZ, 1973), as well as in meat packs (GRECZ *et al.*, 1971). Therefore an understanding of the mechanism of inactivation in Phase 6 is important.

From the mechanistic point of view it is essential to consider at least two important factors contributing to the inactivation of spores in this range:

(i) The highly lethal effect of heat itself, and

(ii) the progressively diminishing contribution of radiation effects because of intensive radical annealment in water at high temperatures.

Under anaerobic conditions the main radical responsible for indirect lethal damage is thought to be the ·OH radical (JOHANSEN, 1965; JOHANSEN & HOWARD-FLANDERS, 1965; BLOCK *et al.*, 1967). In briefly reviewing the radiochemistry of water, SUCHANEK *et al.*, (1969) describe an initial step in which an excited water molecule is split into a hydrogen radical (H·) and a hydroxyl radical (·OH). Subsequently the H· and ·OH may undergo a number of recombinations such as:

$$\cdot H + \cdot OH \rightarrow H_2O \quad (1)$$

$$\cdot H + \cdot H \rightarrow H_2 \quad (2)$$

$$\cdot OH + \cdot OH \rightarrow H_2O_2 \quad (3)$$

Furthermore, they may combine with biopolymers:

$$\text{Biopolymer RH} + \cdot OH \rightarrow \text{Biopolymer R} + H_2O \quad (4)$$

$$\text{Biopolymer } R_1H + \cdot H \rightarrow \text{Biopolymer } R_1 + H_2 \quad (5)$$

Reaction (1) or (2) results in neutral products, while reaction (3) may result in an accumulation of a toxic product, H_2O_2. Of the cellular effects, reaction (4) frequently leads to lethal damage, while reaction (5) appears to have little biological significance (BLOCK *et al.*, 1967).

The work of SUCHANEK *et al.*, (1969) indicates that thermorestoration patterns of spore survival in aqueous systems were based mainly on the availability of ·OH radicals. Under aerobic conditions the thermorestoration of ·OH radicals was not significantly complicated by O_2-derived radicals since they were not thermorestorable. However, the studies of these investigators were inconclusive as to the role of radical processes in Phases 5 and 6, *i.e.*, at relatively high temperatures, in that no distinction was made between radiation effects and the effects responsible for thermal inactivation of the

spores. Annealment of ·OH radicals is increased in proportion to the increase in temperature. Therefore, very few ·OH radicals should be left at highly lethal temperatures.

In order to study the involvement of radicals during the highly lethal temperature range of Phase 6, we have utilized two radical scavengers which are normally common to food products. They are:

(i) 2*M* ethanol, a scavenger of ·OH radicals, and
(ii) 1*M* $NaNO_3$, a scavenger of H· and e^-_{aq} radicals.

The mode of action of these substances on the principal radicals formed by ionizing radiation in aqueous systems is characterized in Table 1.

Table 1

Effect of radical scavengers or lethal radicals of ionizing radiation on spores of B. stearothermophilus (JOHANSEN & HOWARD-FLANDERS, 1965)

Compound	Effective concentration, Moles	Reaction rate constant ($M^{-1}sec^{-1}$)			Protection at 25°C	Senzitization at 25°C
		·H	e^-_{aq}	·OH		
Nitrate, NO_3	1	6×10^6	6.6×10^9 2.2×10^{10}	Nil	—	82%
Ethanol	2	9×10^6	1×10^5	2.5×10^8 2×10^9	77%	—

1. Materials and methods

1.1. Microbiological

Wild type *Bacillus cereus* T from *Dr. H. O. Halvorson*, University of Minnesota and *B. stearothermophilus* NCIB 8224 from *Dr. J. Tramer*, United Dairies Ltd., London, W. 12 were selected on the basis of differences in their heat and radiation resistances. Stock cultures were maintained on nutrient agar slants.

1.2. Spore production, cleaning and chemical state: Bacillus stearothermophilus and Bacillus cereus

The methods of spore production, cleaning and peparation of chemical spore state have been described in an earlier paper (FRIEDMAN & GRECZ, 1973). Only the Ca^{++} form spores were used in the present study.

1.3. Determination of heat resistance

The heat resistance of the spores was determined by dispensing 1.2 ml of the spore suspension into 10×150 mm Pyrex test tubes. The spores were suspended in solutions at $1M$ $NaNO_3$, or $2M$ ethanol, saturated with N_2 by bubbling the gas through each tube for 5 minutes, then sealed in an oxygen flame. While the tube was sealed nitrogen gas was continually blown into the top of the tube to insure saturation of the sample (SUCHANEK *et al.*, 1969).

Heating of the spores of *B. cereus* was done by total submerging of the sealed tubes in a water bath. Spores of *B. stearothermophilus* were submerged in a bath of ethylene glycol. Heated tubes were removed from the bath according to a prearranged experimental time schedule and immersed in a crushed ice-water bath.

Control samples (no irradiation) were exposed to identical conditions, *i.e.*, a come-up time of 2.5 minutes; a 7.4 minute exposure at each temperature (equivalent to the time required for irradiation of irradiated samples), and a rapid cooling in crushed ice-water bath, 3 minutes. Survivors were measured by plating on Tryptone Glucose Yeast Extract Agar (Difco) for spores of *B. cereus* and incubated at 30 °C for 48 hours. *B. stearothermophilus* spore survivors were plated on Dextrose Tryptone Bromocresol Purple Agar (Difco) and incubated at 55 °C for 48 hours.

1.4. Irradiation of spores

Cleaned, heat-shocked spores were suspended in $1M$ $NaNO_3$ or $2M$ ethanol and distributed in 1.2 ml aliquots into 10×75 mm Pyrex tubes. The samples were kept in ice-water during equilibration with nitrogen gas. Anoxic conditions were achieved in the spore suspension by bubbling nitrogen gas through the sample for 5 minutes. While the tube was flame sealed, gas was continually blown into the top of the tube in order to insure N_2 saturation of the sample (SUCHANEK *et al.*, 1969).

The spores were irradiated with ^{60}Co to 0.45 Mrad (dose rate 6.1×10^4 rad min^{-1}). The temperature during irradiation was controlled at $-15°$ to 120 °C in regularly spaced temperature intervals in 5° or 10 °C increments. Come-up time was 2.5 minutes, exposure time 7.4 minutes, cooling 3 minutes. Temperature was maintained within ± 2 °C by a specially constructed blower box (GRECZ *et al.*, 1965).

2. Results

2.1. Heat resistance of selected organisms

The calcium form spores of *B. stearothermophilus* showed at 115 °C a D value (time necessary to reduce spore counts by 90%) of 15 minutes; calcium

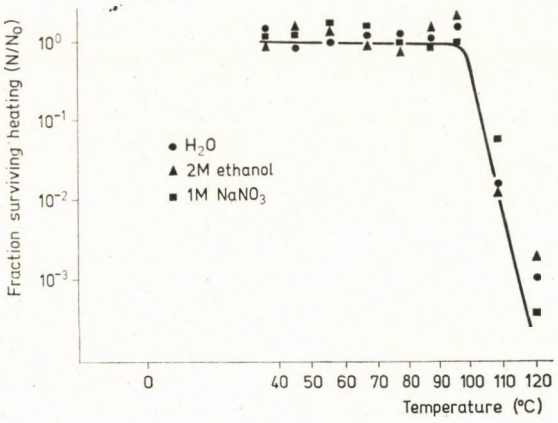

Fig. 2. Effect of heat in the presence of 2*M* ethanol or 1*M* $NaNO_3$ on Ca^{++} form spores of *B. stearothermophilus*. Come up time to the indicated temperature was 2.5 minutes, exposure time 7.4 min (*i.e.* equivalent to the time required to irradiate the irradiated samples), cooling time in crushed ice was 3 minutes. Spores were immediately diluted and plated on agar plates to determine survival

spores of *B. cereus* had a D value at 90 °C of 15 minutes (FRIEDMAN &GRECZ, 1973). Although it has been previously found that 2*M* ethanol and 1*M* $NaNO_3$ have no toxic effect on spores at room temperature, their effect on heat resistance is not known. The results summarized in Fig. 2 and Fig. 3 indicate that 2*M* ethanol as well as 1*M* $NaNO_3$ had no detectable effect on the heat resistance of calcium spores of *B. stearothermophilus* and *B. cereus* under the experimental conditions used for irradiation in the present experiments.

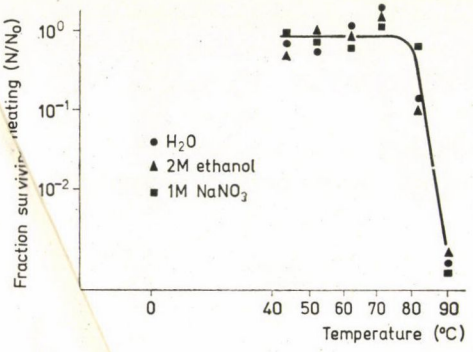

Fig. 3. Effect of heat in the presence of 2*M* ethanol or 1*M* $NaNO_3$ on Ca^{++} form spores of *B. cereus*. The same treatment schedule was employed as described in the caption to Fig. 2

2.2. Radiation resistance

Spores of *B. stearothermophilus* (in the Ca^{++} form) showed a D_{10} value (dose necessary to reduce spore counts by 90%) of 0.12 Mrad, while those of *B. cereus* had a D_{10} value of 0.22 Mrad when irradiated at 0 °C (FRIEDMAN & GRECZ, 1973).

2.3. Scavenging of ·OH radicals by ethanol

The mechanism of radioprotective effect of the ethanol is due to its ability to react with ·OH radicals.

$$\cdot OH + CH_3CH_2OH \rightarrow H_2O + CH_3\,\dot{C}H\,OH \qquad (6)$$

Ethanol is an excellent scavenger of ·OH radicals ($K = 2.5 - 20 \times 10^8\ M^{-1}\ sec^{-1}$) with relatively low affinity for ·H ($K = 9 \times 10^6\ M^{-1}\ sec^{-1}$) and e^-_{aq} ($K = 1 \times 10^5\ M^{-1}\ sec^{-1}$). Therefore, ethanol was chosen to identify and characterize possible involvement of ·OH radical in the steep killing of spores when irradiated at highly lethal temperatures of 105 °—110 °C for *B. stearothermophilus* or 78 °—85 °C for *B. cereus*. The results are summarized in Fig. 4 and Fig. 5.

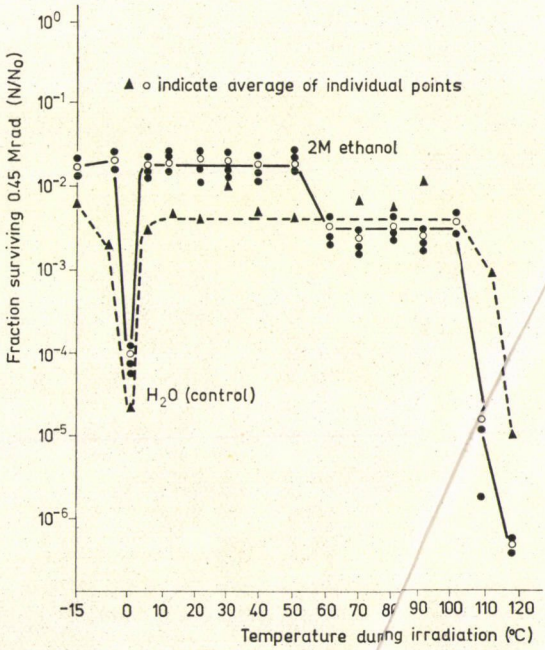

Fig. 4. Effect of 2*M* ethanol and temperature on the radiation survival of Ca^{++} form spores of *B. stearothermophilus* suspended in anoxic water

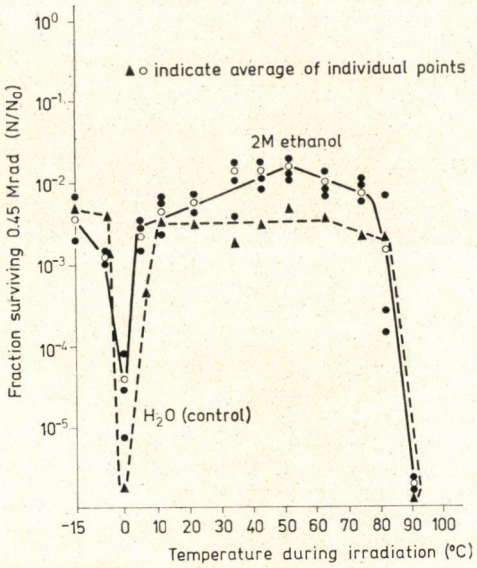

Fig. 5. Effect of 2*M* ethanol and temperature on the radiation survival of Ca^{++} form spores of *B. cereus*

Spores of *B. stearothermophilus* were strongly protected by ethanol at all temperatures between —15 °C and 50 °C (Fig. 4) implicating a major role of ·OH radicals in this temperature range. However, between 60 ° and 100 °C this protection disappeared. The highly lethal effect commenced at 100 °C in 2*M* ethanol as compared with 110 °C in the control containing no ethanol. Spores of *B. cereus* showed a similar, although somewhat less articulated survival pattern (Fig. 5). They were strongly protected by ethanol at all temperatures between 0 ° and 50 °C. Here again, the rapid decline in spore numbers at the terminal end of the survival pattern commenced at a somewhat lower temperature in ethanol (about 55 °C) as compared with the control containing no ethanol (65 °C).

2.4. Scavenging of H· and e^-_{aq} by nitrate

Nitrate is an excellent scavenger of hydrated electrons ($K = 6.6 \times 10^9$ M^{-1} sec^{-1}) and hydrogen radicals ($K = 6.0 \times 10^6$ M^{-1} sec^{-1}). However, nitrate does not react appreciably with hydroxyl radicals. The effects of nitrate on the thermorestoration pattern of spores are given in Fig. 6 and Fig. 7.

Both curves are essentially similar indicating that nitrate strongly enhanced radiation death of spores at all temperatures including the highly lethal range above 70 ° for *B. cereus* and 110 °C for *B. stearothermophilus*.

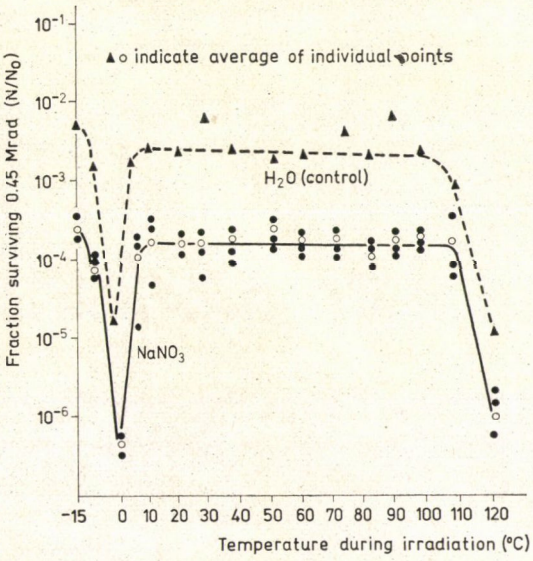

Fig. 6. Effect of 1*M* $NaNO_3$ and temperature on the radiation survival pattern of Ca^{++} form spores of *B. stearothermophilus*

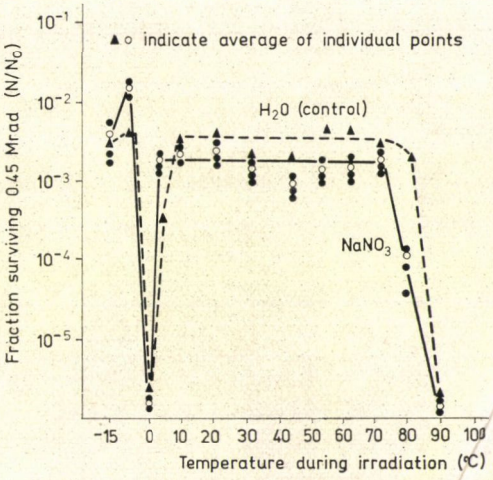

Fig. 7. Effect of 1*M* $NaNO_3$ and temperature on the radiation survival pattern of spores of *B. cereus*

3. Conclusions

3.1. Cellular determinants

In assessing radiation inactivation data particularly in Phases 5 and 6 of the thermorestoration pattern, we should first consider the inherent dynamic changes in the spores themselves (FRIEDMAN & GRECZ, 1973). It is probable

that elevated temperatures introduce changes in the permeability of the coat or membranes of the spores. At these severe temperatures, movement of materials out of the spores (dipicolinic acid, calcium, possibly amino acids and fatty acids) has been described (WALKER & MATCHES, 1965; GRECZ & TANG, 1970; LEVINSON & HYATT, 1971). Furthermore, radiation also causes loss of the same materials including amino acids and DPA (FARKAS & KISS, 1965). Subsequent exchange movements of substances into the spore from the medium, particularly water, would be logically expected. In fact, it has been shown that spores become hydrophilic after being heat killed (GRECZ *et al.*, 1970) accompanied by swelling of the core and by breaks in the plasma membrane although no disappearance of the cortex could be detected by electron microscopy (GRECZ *et al.*, 1974a).

From consideration of coordination chemistry and from electron microscopic evidence (GRECZ *et al.*, 1974b) it appears that 3 molecules of water enter the spore core for each molecule of Ca-DPA lost. Thus it may be conjectured that the precipitous killing rate of spores at high temperatures in Phase 6 may elicit a rather complex chain of events on exposure to radiation plus heat. These considerations support the idea that high radiation resistance of spores as opposed to vegetative bacteria is normally due to their dehydrated impermeable nature. Much additional definitive research is needed to rationalize the processes involved in the destruction of spores by radiation plus heat. The important practical recognition that has emerged from our studies is that normally radiation protective agents such as ethanol (this study) and ground beef (GRECZ *et al.*, 1971) appear not to protect spores under Phase 5 and 6 conditions, a conclusion also implicit from radical chemistry evidence discussed below. The practical advantages of the precipitous loss of resistance of spores when irradiated at high temperatures under Phase 6 conditions to food irradiation technology may be of far reaching significance.

3.2. Effect of radical scavengers on thermorestoration patterns

In discussing the effects of heat, radiation and radical scavengers it should be remembered that scavengers affect lethal efficiency of radiation by reacting with ·OH, H· and e^-_{aq}. Ethanol protects by reacting with harmful ·OH radicals. Nitrate sensitizes probably by withdrawing H· and e^-_{aq} which otherwise would combine with ·OH to produce H_2O. These relationships are summarized in Fig. 8.

H· and e^-_{aq} by themselves under ordinary temperatures are thought not to kill microorganisms such as *Escherichia coli* (JOHANSEN, 1965). Therefore, H· and e^-_{aq} would certainly not be expected to attack the much more radiation resistant bacterial spores. H· and e^-_{aq} normally combine with the

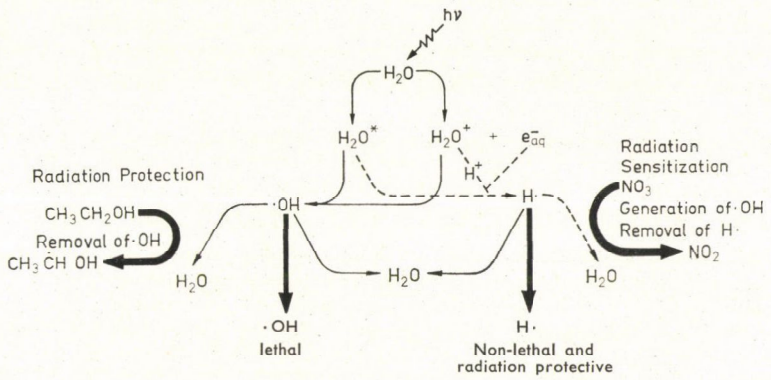

Fig. 8. Radiolysis products of water as affected by ethanol and nitrate. H_2O^* = excited water molecule; $H_2O^+ + e^-$ = ionized water molecule. Hydroxyl radicals · OH are lethal to spores. Hydrogen radicals H · are non-lethal and radiation protective by removal of ·OH radicals. However, H· radicals became lethal to spores when temperature during radiation was > 50°C

harmful ·OH to yield H_2O:

$$e^-_{aq} + H^+ \rightarrow H\cdot \quad (7)$$

$$NO_3 + H\cdot \rightarrow NO_2 + \cdot OH \quad (8)$$

$$H\cdot + \cdot OH \rightarrow H_2O \quad (9)$$

Therefore, by removing H· and e^-_{aq}, nitrate directly (1&2) and indirectly (9) increases the concentration of available ·OH radicals. In this manner nitrate potentiates the destruction of bacterial spores by increasing the concentration of available ·OH in our system.

3.2.1. Quantitative analysis: effect of radiation in the presence of scavengers on the survival of spores. For comparison purposes the data presented in Fig. 4 and Fig. 6 for *B. stearothermophilus* and Fig. 5 and Fig. 7 for *B. cereus* have been recalculated to give Factor F_1. F_1 is distinct from Factor F used previously (FRIEDMAN & GRECZ, 1973):

$$F_1 = \frac{(N/N_0) \text{ for unirradiated spores + heat (control) + Scavenger}}{(N/N_0) \text{ for spores + heat + irradiation + Scavenger}},$$

where N = number of spores surviving a particular treatment, *i.e.*, heat alone, or heat + irradiation; N_0 = initial viable number of spores in the sample. In this formula the effect of heat + scavenger cancel out. The magnitude of F_1 gives a quantitative index of the action of radiation in the presence of the scavenger.

A value of $F_1 = 1$ would indicate no effect of radiation in the presence of scavengers. Any value of $F_1 > 1$ would characterize the contribution of radiation in the presence of a scavenger to spore kill, while $F_1 < 1$ would constitute any effect counteracting the action of radiation, *e.g.*, heat or scavenger.

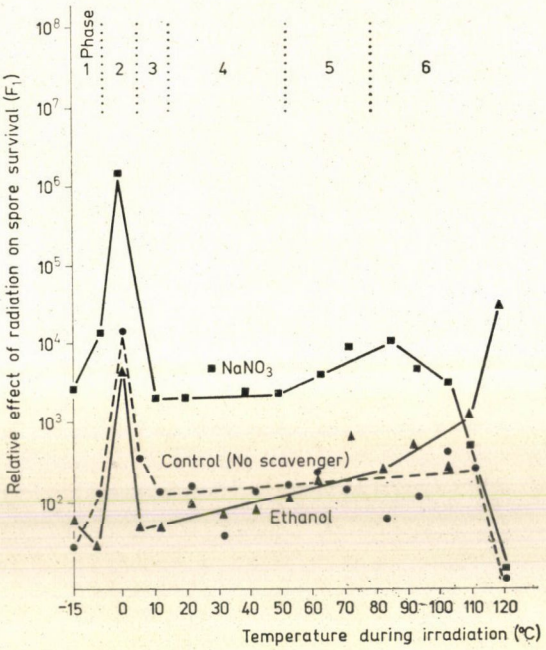

Fig. 9. Effect of radiation in the presence of radical scavengers as compared with the action of radiation without scavenger on the thermorestoration pattern of spores of *B. stearothermophilus*. See para. 3.2.1. for definition of F_1; A value $F_1 = 1$ indicates no effect of radiation, $F_1 > 1$ characterizes the quantitative contribution of radiation, $F_1 < 1$ constitutes protection against radiation by heat or scavenger

3.2.2. Results of calculations of F_1. As shown in Figs. 9 and 10 all our F_1 values were >10, indicating that radiation contributed to spore kill under all conditions in this experiment.

In comparing the two scavengers, ethanol and nitrate, one can deduce the relative contribution of the scavenger from the magnitude of the F_1 values. A further comparison could be made with the control sample containing no scavenger (dotted line in Figs. 9 and 10).

Radiation protection by ethanol and sensitization by nitrate followed expected patterns in Phases 1, 2, 3 and 4 (*viz.*, at −15 °C to +45 °C). However, sensitization by nitrate was more pronounced for: (i) the radiation sensitive *B. stearothermophilus* (Fig. 9), and (ii) at 0 °C where radiation effects were highest.

On the other hand, protection by ethanol was most pronounced for radioresistant *B. cereus* (Fig. 10). However, the protective effect of ethanol appeared to turn into a sensitizing effect, *i.e.*, to reverse itself as temperatures increased to 50 ° to 100 °C for *B. stearothermophilus* and 50 ° to 80 °C for, *B. cereus*. In this temperature range, *i.e.*, in Phases 5 and 6, ethanol caused

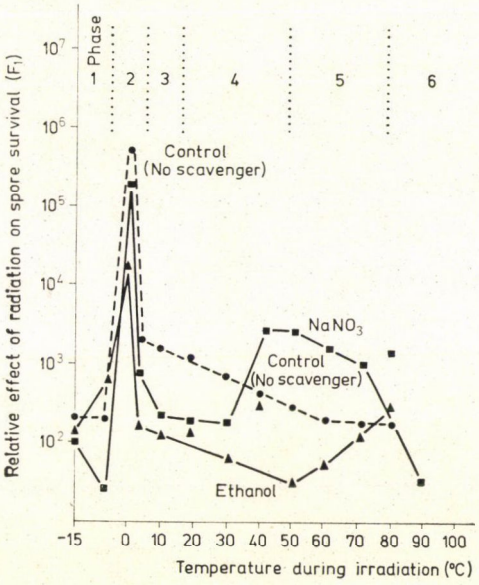

Fig. 10. Effect of radiation in the presence of radical scavengers as compared with the action of radiation without scavenger on the thermorestoration pattern of spores of *B. cereus*. See para 3.2.1. for definition of F_1; A value $F_1 = 1$ indicates no effect of radiation $F_1 > 1$ characterizes the quantitative contribution of radiation, $F_1 < 1$ constitutes protection against radiation by heat or scavenger

distinct increases in spore kill instead of radiation protection. The increase in kill may possibly be attributed to lethality due to ·H radicals which were harmless at lower temperatures. This conclusion was further supported by the fact that nitrate also reversed its effect in approximately the same temperature ranges, so that in Phases 5 and 6 nitrate became radiation protective, instead of sensitizing. This reversal is consistent with the idea that in Phases 5 and 6 the reducing H· and e^-_{aq} radicals became lethal and thus removal of reducing radicals by nitrate offered radiation protection.

It may be conjectured that the apparent reversal of the roles of ·OH and H· radicals in Phases 5 and 6 may represent a change in the predominant spore inactivation mechanism from:

(i) radiation kill in Phases 1, 2, 3 and 4, to
(ii) heat + radiation kill in Phases 5 and 6.

Kill of vegetative cells and spores by ionizing radiations is generally associated with DNA damage such as DNA strand breaks (DURBAN *et al.*, 1974) which in turn is attributed to ·OH radicals (JOHANSEN, 1965; JOHANSEN & HOWARD-FLANDERS, 1965; BLOCK *et al.*, 1967). On the other hand, heat kill is usually related to denaturation of proteins (enzymes and membranes).

Radiation inactivation of biologically active proteins in aqueous systems is usually attributed to both reducing (H·, e^-_{aq}) and oxidizing (·OH) radical species (ALTMAN *et al.*, 1970; EJDUS, 1972). Studies by GRECZ and SEHGAL (1970) with botulinum toxin, a model protein molecule found in *C. botulinum* spores, showed that radiation inactivation of this molecule was accomplished to 48% by H·, 14% by e^-_{aq}, 31% by ·OH and 7% by direct hits. Thus, reducing species H· and e^-_{aq} together contributed a formidable 62% of the inactivation effect. Further studies are in progress probing the role of reducing species with regard to the mechanism of synergistic action of heat and radiation at elevated temperatures. The important question from the practical point of view is why do normally radioprotective substances such as ethanol (this study) or ground beef (GRECZ *et al.*, 1971) lose their radioprotective effect when radiation is carried out above 50 °—70 °C.

*

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INVESTIGATIONS INTO THE POSSIBILITIES OF ENZYMATIC HYDROLYSIS OF CELLULOSE-CONTAINING WASTES

PART III – ENZYMATIC HYDROLYSIS OF CHEMICALLY PRETREATED POPLAR SAWDUST AND CORN COB

I. GAJZÁGÓ, P. ROSE and L. VÁMOS-VIGYÁZÓ

(Received May 18, 1973)

Sawdust and corn cob were pretreated with dilute sulphuric acid or the lignin content of the sawdust was removed and the chemically degraded wastes were then hydrolyzed with cellulase enzymes. In order to improve the efficiency of enzymic hydrolysis the commercial fungal preparations which, in preliminary experiments, proved to be the best, were applied in mixtures, too. To obtain liquors of higher reducing matter content, beside 5% suspensions, 7.5 and 10% suspensions were hydrolysed as well.

It was found that the mixtures of the enzyme preparations produced more reducing substance in the sawdust suspensions predigested with dilute sulphuric acid than any of the preparations individually. However, even with the most efficient mixture (Meicelase P – Serva, 2 : 1), applied at a concentration of 3% related to the solids content of sawdust, only a 13% conversion was achieved during a 16-hour treatment at 40°C and pH 5.0. It is possible to increase the concentration of reducing substances in the suspension by increasing the sawdust concentration, however this does not lead to a substantial increase of the conversion value.

The highest concentration of reducing substance was obtained in a sawdust suspension of 10% and this was 1.1% (w/v).

Under the same conditions corn cob yielded 3.9% (w/v) reducing substance and the value of conversion exceeded 40%.

Of all the pretreatments aimed at delignifying the sawdust the highest enzymic conversion ratio (8.3%) was achieved after applying a 45% sodium hydroxide solution. During this treatment, however, the major part of the hemicelluloses went to waste.

Summing up the results of these experiments it may be said that sawdust does not seem to be suitable for the production, by enzymic hydrolysis, of liquors containing fermentable sugars. It requires drastic pretreatment (75% H_2SO_4, 45% NaOH) and is convertible to a low degree (maximum 26%). Far better results may be achieved with corn cob given a mild treatment (2% H_2SO_4) and yielding 40–42% conversion. The development of an efficient industrial process is, however, possible only with enzymes capable of the easy and rapid hydrolysis of high molecular weight, insoluble cellulose.

In a previous communication (GAJZÁGÓ *et al.*, 1973) the results of various chemical pretreatments required to precede the enzymic hydrolysis of poplar sawdust and corn cob, were reported.

In the course of the preliminary experiments the conversion of 5–8% achieved with sulphuric acid predigestion was found to be the most effective in view of enzymic hydrolysis. This two-step predigestion process required a high proportion of sulphuric acid (VÁMOS-VIGYÁZÓ *et al.*, 1972). To simplify the acid predigestion process and to reduce the sulphuric acid requirement a single step process was developed which also resulted in a conversion of 5–8% (GAJZÁGÓ *et al.*, 1973).

Apart from predigestion with dilute sulphuric acid some methods were tested to delignify the sawdust with hydrochloric acid-dioxan-water mixtures and NaOH solutions of various concentrations in order to make the cellulose and hemicellulose contents available to enzymic hydrolysis.

The predigestion with dilute sulphuric acid of corn cob suspensions of various concentrations was also studied.

Here the results of experiments into the hydrolysis with cellulase enzyme preparations of chemically pretreated sawdust and corn cob are reported.

1. Materials and methods

1.1. Materials

1.1.1. Sawdust predigested with sulphuric acid. Suspensions of 5, 7.5 and 10% (w/v) of the 0.315—1.00 mm fraction of poplar sawdust were pretreated with a 2% sulphuric acid solution at 100 °C for 2 hours. This treatment was necessary to achieve a conversion of 5—8% found most suitable for the efficiency of enzymic hydrolysis (VÁMOS *et al.*, 1970; GAJZÁGÓ *et al.*, 1971). The sawdust suspension thus predigested, then adjusted to pH 5.0 with ammonium hydroxide, was hydrolyzed with enzyme preparations.

1.1.2. Corn cob predigested with sulphuric acid. Corn cob suspensions of 5 and 10% (w/v), respectively, were predigested in a 2% sulphuric acid solution for 2 hours at 100 °C. About 30% of the solids content were converted to reducing matter. Suspensions thus obtained were adjusted to pH 5.0 and then exposed to enzymic hydrolysis.

1.1.3. Sawdust pretreated with hydrochloric acid-dioxan-water mixtures. In order to reduce the lignin content various mixtures of 5*N* hydrochloric acid, dioxan and water were applied to 5% (w/v) suspensions of sawdust at 92 °C for 3 hours. The sawdust was then washed with 500-fold of its volume of hot, and then the same amount of cold, distilled water, dried, and made up to a 5% (w/v) suspension with phosphate-citrate buffer of pH 5.0. This suspension was used for enzymic hydrolysis.

1.1.4. Poplar sawdust pretreated with NaOH solutions. Sawdust suspensions of 5% (w/v) were treated with NaOH solutions of 1, 3, 5, 15 and 45% (w/v), respectively, at 100 °C for 3 hours. The suspensions thus treated were then washed according to para. 1.1.3., dried and resuspended in the buffer solution. The suspension thus obtained was exposed to enzymic hydrolysis.

1.1.5. Enzyme preparations used. Three enzyme preparations found most suitable in the course of the preliminary experiments (VÁMOS *et al.*, 1970) were applied. The names of these preparations and their filter paper degrading activity, determined by the method of NIWA and NAKAMURA (1965), are given in Table 1.

Table 1

Characteristics of the enzyme preparations applied in sawdust hydrolysis

Preparation	Manufacturer	Microorganism used for production	Enzyme concentration of the preparation	
			$U \cdot mg^{-1}$*	s
Merck Cellulase	E. Merck A. G. Darmstadt, F. R. G.	unknown	230	15
Macerozyme	All Japan Biochemicals Co. Ltd., Nishinomiya, Japan	*Rhizopus* sp.	125	7
Meicelase P	Meiji Seika Kaisha Ltd., Tokyo, Japan	*Trichoderma viride*	188	10
Serva Cellulase	Serva Entwicklungslabor, Heidelberg, F. R. G.	*Aspergillus niger*	93	5

* The preparation is of unit enzyme concentration if 1 mg of it liberates 1 μg of glucose from a 1% suspension of Macherey and Nagel MN 640 filter paper at pH 5 and 40°C in 60 minutes
s = standard deviation
n = 5—6

1.2. Methods

1.2.1. Enzymic hydrolysis.

1.2.1.1. Enzymic hydrolysis after pretreatment with dilute sulphuric acid. — Suspensions of sawdust or corn cob pretreated with dilute sulphuric acid were adjusted to pH 5.0. Enzyme preparations or their mixtures were added at a rate of 3% as related to solids of cellulosic material.

In some of the experiments, apart from increasing the sawdust concentration of the suspension, the enzyme concentration related to the volume of the suspension was kept at a constant level (1.38 $mg \cdot ml^{-1}$). This value, related to the solids content of the sawdust suspension, was as follows:

Sawdust concentration in the suspension, % (w/v)	Concentration of enzyme preparation on dry matter basis, %
5.0	3.00
7.5	2.25
10.0	1.50

An 8-place thermostat provided with agitators of 180 rpm was used for the enzymic hydrolysis. At the end of the hydrolysis period the samples

were placed into a cold room of —20 °C temperature in order to stop the enzyme reaction and to prevent microbial spoilage. Hydrolysis periods of 4, 8 and 16 hours, respectively, were applied at 40 °C. In the course of the preliminary experiments (VÁMOS-VIGYÁZÓ *et al.*, 1972) the results obtained at 40, 50 and 60 °C were found to be identical and thus it seemed expedient to choose the lowest temperature.

1.2.1.2. Enzymic hydrolysis after various pretreatments. — After pretreatment with hydrochloric acid-dioxan-water mixtures or NaOH solutions suspensions were prepared according to paras. 1.1.3. and 1.1.4. 3% of the enzyme mixture Meicelase P — Serva (2 : 1), related to the pretreated raw material, was added. Hydrolysis was carried on for 8 hours under the conditions and in the equipment as described above.

1.2.2. Measurement of the reducing substance. The reducing substances formed during enzymic hydrolysis were determined by the method of SOMOGYI (1952) and were expressed as glucose.

Prior to the determination of the reducing matter protein was removed from the filtered liquors (BALDWIN *et al.*, 1953).

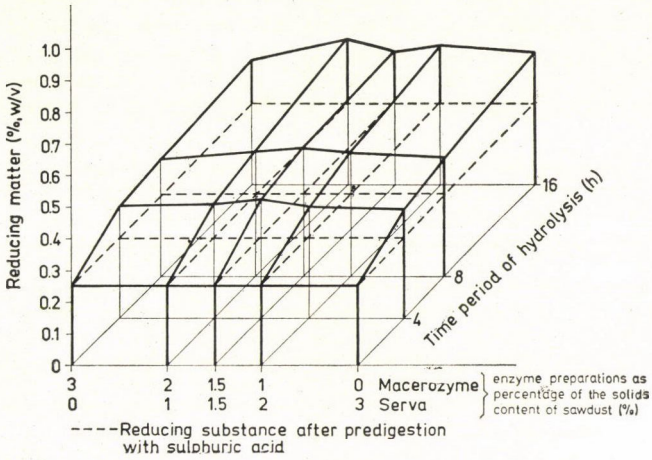
1.2.3. Mathematical statistical analysis of the results. Student's *t* test was applied for statistical evaluation. The significance levels of the differences between values compared (KÖRMENDY, 1964) are shown in the figures. Significance levels of 95, 99 and 99.9% were marked x, xx and xxx, respectively, and Ø or — was used to mark differences not significant.

2. Results

2.1. Enzymic hydrolysis of poplar sawdust predigested with sulphuric acid

Changes in reducing matter during enzymic hydrolysis of 5% (w/v) sawdust suspensions, predigested with 2% sulphuric acid, are illustrated in Figs. 1—3.

As can be seen, the progress in time of hydrolysis is similar with every enzyme preparation or their mixtures: the rate of reducing sugar formation is highest in the first 4 hours. All the enzyme mixtures were more efficient than any of the preparations individually. Serva cellulase, in itself of poor hydrolyzing capacity, is very efficient in complementing the effect of the other enzymes. The highest reducing matter content achievable with the 2 : 1 mixture of Meicelase P and Serva enzyme preparations is very highly significantly higher than the best results obtained with the two other enzyme mixtures (Merck—Serva, 1 : 1, Macerozyme—Serva, 2 : 1). With the 2 : 1 mixture of Meicelase P and Serva reducing matter in the predigested suspension was nearly doubled during the 16-hour hydrolysis period. However, the com-



Significance level of differences

Hydrolysis period (h)	Macerozyme-Serva, 2:1				Macerozyme-Serva, 1:1				Macerozyme-Serva, 1:2				Macerozyme				Serva			
	0	4	8	16	0	4	8	16	0	4	8	16	0	4	8	16	0	4	8	16
Macerozyme-Serva 2:1																				
0		xxx	xxx	xxx		xxx	xxx	xxx		xxx	xxx	xxx		xxx	xxx	xxx		xxx	xxx	xxx
4			—	xxx		—				—				—				x		
8				xx			—				—				—				—	
16								xxx				—				xxx				xx
Macerozyme-Serva 1:1																				
0					xxx	xxx	xxx													
4						xxx	xxx			—				xxx				xxx		
8							xx				—				xx				xx	
16												xxx				xx				—
Macerozyme-Serva 1:2																				
0									xxx	xxx	xxx									
4										xxx	xxx		—					—		
8											xx			—					x	
16															xxx					x
Macerozyme																				
0													xxx	xxx	xxx					
4														xx	xxx			—		
8															xxx				—	
16																				—
Serva																				
0																		xxx	xxx	xxx
4																			xxx	xxx
8																				xx
16																				

Fig. 1. Effect of the composition of the Macerozyme — Serva enzyme mixture and the time of treatment upon the hydrolysis of a 5% (w/v) sawdust suspension predigested with sulphuric acid. Experimental conditions: 5% (w/v) sawdust suspension predigested with 2% sulphuric acid solution for 2 hours at 100°C, adjusted to pH 5.0 with NH_4OH, exposed to enzymic hydrolysis at 40°C with 3% enzyme preparation as related to dry matter of sawdust. Agitated at 180 rpm. Number of parallel experiments, n = 6

bined effect of acid and enzymic hydrolysis amounted to 11% conversion, only.

Changes in reducing matter, in sawdust suspensions of 5, 7.5 and 10% (w/v) as an effect of the 2 : 1 mixture of Meicelase P and Serva preparations, added to the suspension at 3% concentration on dry matter basis are shown in Fig. 4.

The reducing substance concentration in the 7.5% suspension increased at a higher rate than in the 5% suspension. The total reducing substance

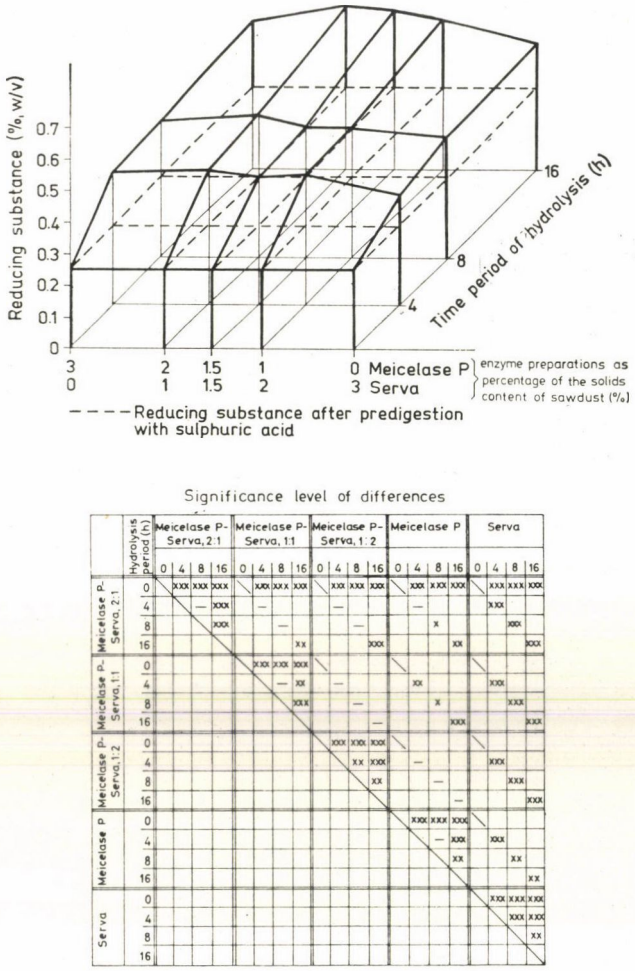
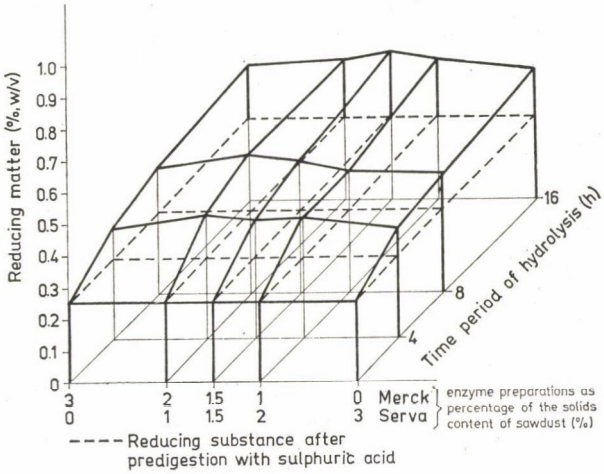


Fig. 2. Effect of the composition of the mixture of enzyme preparations Meicelase P and Serva and of the treatment time upon the hydrolysis of a 5% (w/v) sawdust suspension predigested with sulphuric acid. For experimental conditions see legend to Fig. 1

content of the 10% suspension is higher at every point of measurement than in either of the suspensions of lower concentrations, however, the amount of sugar formed by the enzyme is less than in the 7.5% suspension. The ratio of the total reducing substance formed on enzymic and acid hydrolysis, respectively, is in the sequence of sawdust concentrations in the suspension, as follows: 0.85 : 1, 1.19 : 1, 0.81 : 1.

About 1% reducing matter was achieved in the 7.5% suspension in about a 16-hour hydrolysis period, while in the 10% suspension about 4 hours were sufficient to obtain the same concentration. The conversion value obtained



Significance level of differences

	Hydrolysis period (h)	Merck-Serva, 2:1				Merck-Serva, 1:1				Merck-Serva, 1:2				Merck				Serva			
		0	4	8	16	0	4	8	16	0	4	8	16	0	4	8	16	0	4	8	16
Merck-Serva, 2:1	0	\	xxx	xxx	xxx	\	xxx	xxx	xxx	\	xxx	xxx	xxx	\	xxx	xxx	xxx	\	xxx	xxx	xxx
	4		\	xx	xxx		\	–			\	–			\	xx			\	xx	
	8			\	–			\	–			\	–			\	xx			\	xxx
	16				\				\				\				\				\
Merck-Serva, 1:1	0					\	xxx	xxx	xxx					\				\			
	4						\	xxx	xxx		\	–			\	xx			\	xx	
	8							\	xx			\	x			\	–			\	xxx
	16								\				\				\				\
Merck-Serva, 1:2	0									\	xxx	xxx	xxx	\				\			
	4										\	–	xxx		\	x			\	xx	
	8											\	xxx			\	–			\	xx
	16												\				\				\
Merck	0													\	xxx	xxx	xxx	\			
	4														\	xxx	x		\	–	
	8															\	x			\	xx
	16																\				\
Serva	0																	\	xxx	xxx	xxx
	4																		\	xxx	xxx
	8																			\	xx
	16																				\

Fig. 3. Effect of the composition of the mixture of enzyme preparations Merck and Serva and of the treatment time upon the hydrolysis of a 5% sawdust suspension predigested with sulphuric acid. For experimental conditions see legend to Fig. 1

in a 16-hour hydrolysis was 13.4% for the 7.5% suspension and 12.3% for the 10% suspension.

In experiments where enzyme concentration related to volume of suspension was kept constant [0.138% (w/v)] a reducing substance concentration of 0.23% (w/v) was obtained in the 5% suspension, 0.46% (w/v) in the 7.5% suspension and 0.38 (w/v) in the 10% suspension. Thus the reducing substance concentration produced by enzyme action and the pertinent conversion value are highly significantly higher in the 7.5% suspension than in both other

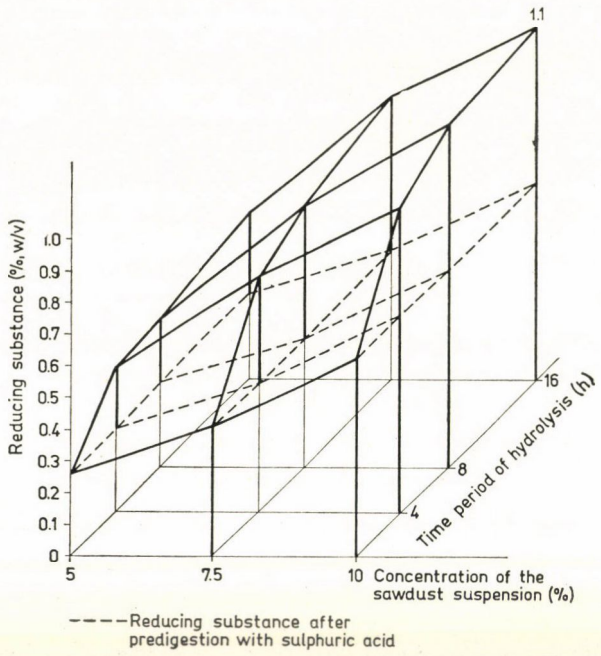


Fig. 4. The amount of reducing substances formed, as affected by the reaction period and the concentration of the sawdust suspension, upon the application of the 2 : 1 mixture of enzyme preparations Meicelase P and Serva. Conditions of treatment: sawdust suspensions predigested with 2% sulphuric acid solution for 2 hours at 100°C, adjusted to pH 5.0 with NH_4OH, exposed to enzymic hydrolysis with 3% enzyme preparation on dry matter basis, at 40°C. Agitated at 180 rpm. n = 6

suspensions. The results obtained in suspensions of three different concentrations with equal enzyme concentrations as related to the volume and to the dry sawdust content of the suspensions, respectively, are compared in Table 2.

2.2. Enzymic hydrolysis of corn cob predigested with dilute sulphuric acid

Corn cob suspensions of 5 and 10% (w/v) predigested with 2% sulphuric acid for 2 hours were hydrolyzed with a 2 : 1 mixture of Meicelase and Serva enzyme preparations applied in a 3% concentration as related to corn cob solids. Hydrolysis was carried on for 4, 8 and 16 hours, respectively. The change in reducing substance concentration, as caused by enzymic hydrolysis is shown in Fig. 5.

The reducing substance concentrations of the 5 and 10% (w/v) corn cob suspensions, predigested with dilute sulphuric acid, were increased by about 50% during the 16-hour treatment and amounted to 2.8 and 3.9% (w/v), respectively. Thus, about 43.6 and 41.8% of the total solids content

Table 2

Hydrolysis of sawdust suspensions of different concentrations by identical enzyme concentrations (a) related to the volume of the suspension, (b) related to the solids content of the sawdust

Sawdust in the suspension (%, w/v)	Enzyme concentration		Reducing substance produced by the enzymes		Level of significance of differences
	in the suspension (%, w/v)	as percentage sawdust solids %	(%, w/v)	s	
5.0	0.138	3.0	0.23	0.01	—
7.5	0.138	2.25	0.46	0.01	***
	0.207	3.0	0.50	0.01	
10.0	0.138	1.5	0.38	0.02	***
	0.276	3.0	0.50	0.02	

Experimental conditions: Predigestion with 2% (w/v) sulphuric acid for 2 hours at 100°C. Adjusted to pH 5.0 with NH_4OH, hydrolyzed with the 2 : 1 mixture of Meicelase P and Serva enzymes for 16 hours at 40°C

s = standard deviation

*** = significance of difference at the probabiiity level of 99.9%

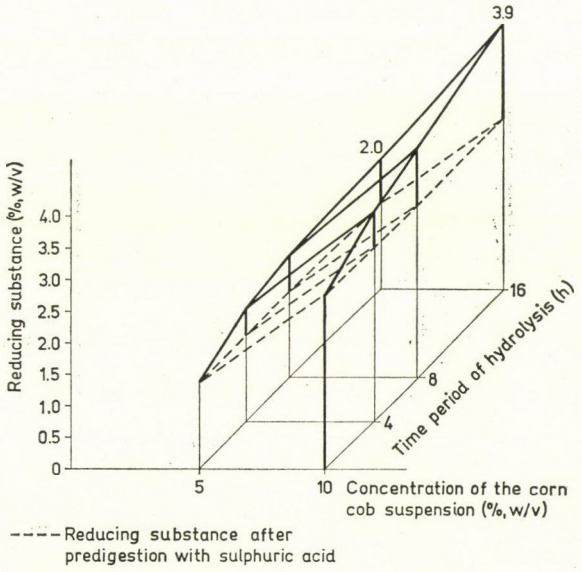


Fig. 5. The amount of reducing substances formed, as affected by the reaction period and the concentration of the corn cob suspension, upon the application of the 2 : 1 mixture of enzyme preparations Meicelase P and Serva. Experimental conditions: corn cob suspensions predigested with 2% sulphuric acid solution for 2 hours at 100°C, adjusted to pH 5.0 with NH_4OH, hydrolyzed with 3% enzyme preparation on dry matter basis, at 40°C. Agitated at 180 rpm. n = 6

were converted to reducing substance of which 13.2 and 12.0%, respectively, were converted by the enzyme. There is no significant difference between these two values.

2.3. *Enzymic hydrolysis of poplar sawdust predigested with hydrochloric acid-dioxan-water mixtures*

Of the HCl-dioxan-water mixtures described in an earlier paper (GAJZÁGÓ *et al.*, 1973) the 1 : 9 and 2 : 3 mixtures of 5*N* HCl and dioxan were found to be most efficient with respect to delignification of poplar sawdust. Both mixtures solubilized about 46—47% of the dry sawdust, *i.e.* more than the double of the lignin content of approximately 22% (NIKITIN, 1955).

From the point of view of enzymic hydrolysis the 1 : 9 mixture seemed to be more efficient, however, the result achieved was still very low. A con-

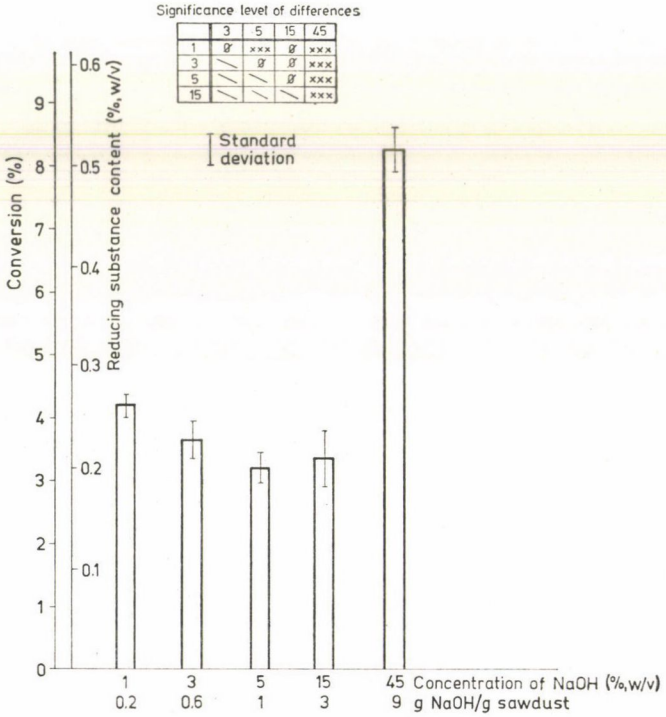


Fig. 6. The effect of the concentration of NaOH used to remove the lignin content of sawdust or of its specific amount upon the availability of the residue to hydrolysis by the 2 : 1 mixture of Meicelase P and Serva. Experimental conditions: sawdust predigested with NaOH at 100°C for 3 hours, washed with distilled water, dried at 105°C, the residue suspended in a phosphate — citrate buffer of pH 5.0, hydrolyzed with 3% enzyme mixture on dry matter basis, at 40°C for 8 hours. Agitated at 180 rpm. n = 5

version of about 4% (0.25% w/v reducing substance) was obtained with the 2 : 1 Meicelase P — Serva mixture during 8 hours in the suspension thus pretreated.

2.4. Enzymic hydrolysis of sawdust predigested with NaOH

Fig. 6 shows the correlation between NaOH concentration or specific amount, used to reduce lignin content, on the one hand, and the conversion value or reducing substance concentration, achieved by an 8-hour treatment with the 2 : 1 enzyme mixture Meicelase P — Serva, on the other.

In spite of the fact that about the same portion of sawdust solids (about 20%) was solubilized with 1% and 45% NaOH, respectively, from the point of view of subsequent enzymic hydrolysis the higher NaOH concentration proved more advantageous: a doubled conversion value, though altogether only 8%, could be achieved under identical conditions.

3. Conclusions

3.1. Enzymic hydrolysis of poplar sawdust predigested with sulphuric acid

All the enzyme mixtures tested in the experiments, and particularly the 2 : 1 mixture of Meicelase P and Serva preparations were more efficient than the individual enzyme preparations (Figs. 1, 2, 3). The mixture of the enzymes of *Trichoderma* and *Aspergillus* origin (Fig. 2) gave the best results probably because of the complementing effect of these two preparations. Cellulase obtained from *Trichoderma* degrades crystalline and amorphous cellulose, while the *Aspergillus*-enzyme acts on soluble derivatives and cellobiose (TIUNOVA *et al.*, 1970). However, the best result, achieved in a sawdust suspension of 5% (w/v) (11% conversion, 0.51% (w/v) reducing substance) is extremely poor in comparison with earlier results obtained by non enzymic processes (KOBAYASHI, 1971).

By increasing the sawdust concentration in the suspension under otherwise identical conditions, the concentration of reducing substances formed upon combined acid and enzyme treatment, may be increased, but the difference is not substantial (Fig. 4). This permits of the conclusion that only part of the predigested sawdust serves as substrate to the enzyme preparations.

Comparing enzymic hydrolysis of 5, 7.5 and 10% (w/v) sawdust suspensions it can be seen that with 3%, on a dry matter basis, of the 2 : 1 mixture of Meicelase P and Serva preparations 41.1, 53.8 and 35.7%, respectively, of the total reducing substance formed by combined acid and enzymatic hydrol-

ysis, was due to enzyme action. Thus, with the given enzyme concentration the sawdust concentration of 7.5% was most advantageous from the point of view of enzymic hydrolysis. The lower result obtained in the 10% suspension is probably due to the higher density or worse mixing characteristics of this suspension.

By increasing the sawdust concentration of the suspension from 5 to 7.5% (w/v) the amount of reducing substance formed as an effect of the same enzyme concentration, was doubled. The increase of the reducing substance concentration is even more extensive if the enzyme concentration is increased in proportion to the substrate concentration, or in other words, the specific enzyme utilization is maintained at a constant level (Table 2).

In the preliminary experiments (VÁMOS-VIGYÁZÓ *et al.*, 1972) predigestion of sawdust suspensions in two steps with sulphuric acid of higher concentration and enzymic hydrolysis, similar to that described in this paper, resulted in 26.5% conversion of solids content as a maximum. In the experiments reported here only 13% conversion could be achieved with dilute sulphuric acid and enzyme treatment. It seems that predigestion with dilute sulphuric acid, though producing a 5—8% conversion, found optimal in the two-step predigestion with more concentrated sulphuric acid (75%), does not degrade the lignin structure of wood sufficiently to make its cellulose, or hemicellulose content available to enzymic hydrolysis. This is evidenced also by the different progress in time of the enzymic hydrolysis of the two suspensions exposed to different pretreatments (VÁMOS-VIGYÁZÓ *et al.*, 1972). Enzymic hydrolysis is highly dependent on the penetration of cellulase between cellulose fibres (SELBY *et al.*, 1963).

3.2. Enzymic hydrolysis of corn cob predigested with sulphuric acid

Corn cob lends itself far better to digestion by dilute sulphuric acid, than poplar sawdust. In contrast to a 5—8% conversion achieved in sawdust suspensions under the same conditions, 29—30% conversion of corn cob was achieved in sulphuric acid digestion. This amounted to 1.41% (w/v) reducing sugar in the 5% suspension and to 2.77% (w/v) in the 10% suspension (Fig. 5).

During the 16-hour hydrolysis period the reducing substance content of the suspension increased only 1.3—1.4-fold as an effect of the enzyme mixture. The low efficiency of the enzymic hydrolysis is probably due to a polysaccharide content extensively reduced by the predigestion, thereby providing a medium for the enzymic hydrolysis of lower substrate concentration and of less advantageous composition.

Of all the experiments described here the best result was achieved in a corn cob suspension of 10% (w/v) by a 16-hour enzymic hydrolysis (3.9%, w/v) (Fig. 5).

3.3. Enzymic hydrolysis of poplar sawdust predigested with a HCl-dioxan-water mixture or NaOH

The mixtures of HCl, dioxan and water did not prove suitable to remove lignin from sawdust prior to enzymic hydrolysis. One of the reasons for this may be that certain substrates of the enzyme are removed with the lignin, such as the easily hydrolyzable hemicelluloses, as shown by the weight loss in excess of the lignin content. On the other hand, with treatments causing lower weight loss a substantial part of the lignin content is retained (GAJZÁGÓ *et al.*, 1971).

Concerning the alkaline predigestion it is of interest to note that — though an equal proportion of sawdust solids was solubilized (17—18%) by both the 1 and the 45% solutions of NaOH (GAJZÁGÓ *et al.*, 1973) — the enzyme produced under identical conditions double amount of reducing substance from the suspension predigested with 45% NaOH (Fig. 6). The reason is probably again the same as in the case of predigestion with sulphuric acid of different concentrations: in spite of the apparently identical result (conversion or loss of weight) wood structure is less affected by the more dilute solution and thus less available to the enzyme.

In the case of alkaline pretreatment, similarly to the pretreatment with HCl-dioxan mixture, the hemicelluloses are removed together with the lignin and probably this is the reason for achieving only a maximum of 8.3% conversion by enzymic hydrolysis.

Thus it may be concluded that of all the methods tested for the predigestion of sawdust the use of sulphuric acid proved to be the most suitable.

The 5—8% conversion, achieved in the preliminary experiments in two steps by treatment with concentrated sulphuric acid, could be achieved in the experiments here reported with dilute sulphuric acid applied in one step. However, the latter method was not suitable from the point of view of the efficiency of enzymic hydrolysis. The combined effect of predigestion with 75% sulphuric acid in two steps and enzymic hydrolysis was a 26.5% conversion of sawdust solids, while predigestion with dilute sulphuric acid in one step and enzymic hydrolysis under otherwise identical conditions, brought about only an 11% conversion of the solids content.

The application of the enzyme preparations in mixtures was more effective than when used individually. It appears from the results, however, that the enzyme preparations used are not particularly suitable to degrade high molecular, insoluble cellulose, even after complete removal of the lignin content. This view seems to be verified by the latest investigations in which after the selective removal of lignin with white-rot fungi (KIRK & MOORE, 1972) as well as after vibro-mill comminution (PEW & WEYNA, 1962; MOORE *et al.*,

1972) a very high percentage of enzyme (75% of the sawdust solids) and 48-hour digestion was necessary to achieve conversion of about 50%.

Corn cob seemed far more available to hydrolysis by acid than sawdust. The application of the same parameters (2% sulphuric acid, 2-hour digestion, at 100 °C) to this raw-material as to sawdust brought about excessive hydrolysis. The moderation of these conditions would have probably resulted in a more advantageous effect of the enzyme mixture.

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METHODS FOR MEASURING MACERASE ACTIVITY

PART I. – INSTRUMENTAL DETERMINATION OF MACERASE ACTIVITY

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(Received May 19, 1973)

To measure macerase activity by its degradative action on plant tissue, a simple device was constructed by means of which it is possible to subject plant tissues always to the same pressure. A copper rod of 80 g weight and 2 mm lower diameter was placed on 1 mm thick potato slices held between plastic rings in a test tube and the enzyme solution pipetted into the tube. During the incubation period the enzyme solution destroys the cell walls and the potato slice collapses. The point when the metal rod of standard weight ruptured the slice of potato and fell into the test tube was considered as the end of the reaction.

This method was found to be sufficiently sensitive and well reproducible for the determination of macerase activity.

With the use of the above method the pH optimum of macerase activity (of our inductive polygalacturonase preparations PG-C_1 and PG-179) was found to be 3.0 in the temperature range tested (20–50°C). The macerase activity could be increased by raising the incubation temperature but temperatures above 40°C cannot be recommended because of the danger of denaturation of the potato tissue.

In case of the above inductive polygalacturonase preparations obtained from *Aspergillus foetidus*, the optimum pH (3.0) for maceration was in agreement with the pH optimum of endo-polygalacturonase. At an incubation temperature of 40°C extreme values were found at pH 4.0, 4.5 and 5.0, where pH 4.0 agrees with the pH optima of the enzymes of xylanase and arabanase, pH 4.5 with the pH optimum of apple-juice-clarifying enzyme component and pH 5.0 with the optimal pH of pectin-transeliminase.

Macerase (macerating enzyme) was first described in the work of phytopathologists (SINGH & WOOD, 1956; LAPWOOD, 1957) who claimed that the first step in the attack of phytopathogenic microorganisms is the destruction of the intact surface of plant tissues by macerase whereby the way is made accessible to the effect of toxins.

Earlier the macerating enzyme was called cytase and again protopectinase (TRIBE, 1955). Several authors have tried to identify the macerating enzyme as some other enzyme, *e.g.* WOOD (1955) who believed it to be the same as protopectinase, while others (BELL *et al.*, 1950; LUH & PHAFF, 1954) thought that it might be identical with polygalacturonase, specifically with endo-polygalacturonase. This last statement was refuted by BYRDE and FIELDING (1965) who were able to separate endo-polygalacturonase from macerase by means of gel filtration. The same authors assumed the identity of the macerating enzyme with alpha-L-arabinofuranosidase.

KAJI and his team (1966) reported on two pH optima of macerase, namely at pH values 3.0 and 5.0, and attributed the second to the overall action of endo-polygalacturonase (endo-PG) and pectin-methylesterase.

BYRDE and FIELDING (1968) found a highly significant correlation at pH 5.0 to 5.2 between the activities of pectin-transeliminase and of macerase which is in agreement with the observations of BUSH and CODNER (1968).

There is a method described in the literature (mainly in the publications of Japanese authors) for the determination of the enzyme activity of macerase. Essentially this method consists in measuring the time needed for the collapse of the plant tissue when immersed in the solution of the enzyme. Changes in the elasticity of the plant tissue up to its complete collapse were tested by compression with a glass rod. These tests are, however, rather subjective, since it is impossible to ensure identical compressing by means of manual force.

In this paper we wish to report on the results of the work concerning the determination of the temperature and pH optima of the macerating enzyme and the effect of enzyme concentration.

1. Materials and methods

1.1. Instrumental test method

The soft tissue consisting of parenchymal cells of potato tubers was used as substrate. The potato was always the *Gülbaba* variety. With the help of the microtome and a cork-borer slices of 100 mm diameter and 1.0 mm thickness were prepared from the potato, the slices placed on plastic cylinders at the bottom of 15-ml test-tubes and fixed with a second similar plastic cylinder.

The test-tubes were arranged on a special stand and 5 ml of enzyme solution were pipetted into each at a quick rate. Finally, copper rods of 80 g weight and 2 mm lower diameter, provided with a plastic cap, were placed on the potato slices between the plastic cylinders.

During the incubation period the enzyme solution destroyed the tissues and as their firmness was lost the copper rod of standard weight penetrated the potato disc and fell into the test-tube. This moment was considered the end of the "reaction". Samples which failed to become destroyed within an incubation period of 3 hours were considered as lacking in macerase activity. The schematic diagram of the method is shown in Fig. 1, the device itself in Fig. 2.

Fig. 1 shows the plastic cylinders in the test-tubes with the slices of potato between them and the copper rods on the discs at the beginning of the

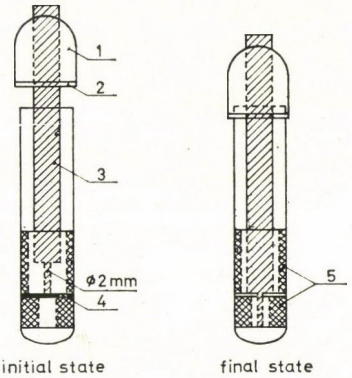

Fig. 1. Schematic diagram of the apparatus constructed at the Central Food Research Institute for the determination of macerase enzyme activity. 1 — plastic cap: 2 — metallic contact; 3 — copper rod; 4 — 1 mm thick slice of potato; 5 — plastic cylinder

reaction and when reaction has been completed, namely when the copper rods have already torn through the potato.

Fig. 2 is the photograph of this simple device. The stand holds 20 test-tubes and can be placed into a thermostat of the desired temperature. At the end of the reaction time the copper rings on the lower edge of the plastic cap will fall on the stand and close a circuit which in turn switches on the corresponding lamp fixed to the back panel of the stand.

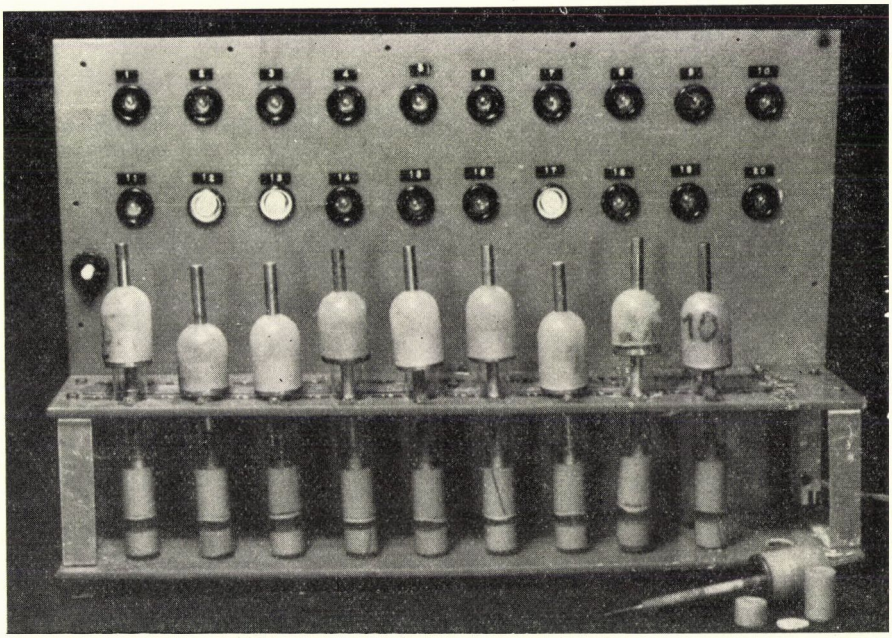

Fig. 2. Apparatus for the determination of macerase enzyme activity

1.2. Enzyme preparations

1.2.1. PG-C_1, inductive polygalacturonase. Liquid preparation produced by submerged fermentation of an *Aspergillus foetidus* strain (Central Food Research Institute, pilot plant production, ZETELAKI-HORVÁTH, 1972). According to our measurements (ZETELAKI & VAS, 1972) the concentrations of the polygalacturonase enzyme components in the liquid preparation were as follows: endo-polygalacturonase, $SPA_{75}^{Na\text{-}pectate}$ 1 670 $l \cdot h^{-1} \cdot l^{-1}$ (substrate: sodium pectate); endo-polymethylgalacturonase, SPA_{75}^{Pom} 327 $l \cdot h^{-1} \cdot l^{-1}$ (substrate: Pomosin pectin, degree of esterification 70%), apple-juice-clarifying polygalacturonase, SPA_{75}^{A} 1 800 $l \cdot h^{-1} \cdot l^{-1}$ (substrate: Jonathan apple-juice of specific viscosity 1.0 and pH 3.8).

1.2.2. Polygalacturonase powder, No. PG-179. The powder is prepared from the culture filtrate of the submerged fermentation of *Aspergillus foetidus* by means of precipitation with methanol (Central Food Research Institute, pilot plant production). Endo-polygalacturonase, $SPA_{75}^{Na\text{-}pectate}$: 94 200 $l \cdot h^{-1} \cdot kg^{-1}$; endo-polymethylgalacturonase, SPA_{75}^{Pom}: 22 600 $l \cdot h^{-1} \cdot kg^{-1}$, apple-juice-clarifying polygalacturonase, SPA_{75}^{A} 288 000 $l \cdot h^{-1} \cdot kg^{-1}$.

1.2.3. Enzyme preparation Pectinol R-10 (Rohm and Haas, Philadelphia, Pennsylvania). Endo-polygalacturonase, $SPA_{75}^{Na\text{-}pectate}$ 477 000 $l \cdot h^{-1} \cdot kg^{-1}$, endo-polymethylgalacturonase, SPA_{75}^{Pom} 36 000 $l \cdot h^{-1} \cdot kg^{-1}$, apple-juice-clarifying polygalacturonase, SPA_{75}^{A} 1 300 000 $l \cdot h^{-1} \cdot kg^{-1}$ (results of the author's determinations).

1.2.4. Determination of enzyme activity. Determination of the polygalacturonase activity of the enzyme preparations was based on the measurement of the viscosity decrease of the substrates (ZETELAKI & VAS, 1972).

1.3. Mathematical statistical methods

The various parameters were compared and the standard deviations of the results determined by means of mathematical statistical methods (WEBER, 1972). When investigating the effect of individual parameters the apparently optimum pH, temperature and enzyme concentration values were compared to the other investigated parameters with the help of the t test and their significance levels determined.

2. Results

2.1. Temperature and pH optimum of the macerating enzyme

With the help of the above described device the temperature and pH optima for the macerating effect of inductive polygalacturonase, type PG-C_1, in the liquid preparation were determined by adjusting its pH to the desired

value with the addition of hydrochloric acid or caustic soda, as required. The time needed for the collapse of the potato tissue was measured in minutes. Since in an enzyme solution of higher activity the plant tissues will hydrolyze within a shorter period of time, a shorter reaction period will correspond to a higher enzyme activity. To facilitate the illustration of this point, that is, in order to characterize a higher enzyme activity with a greater numerical value, the results are given in reciprocal minutes ·100 and are plotted as such

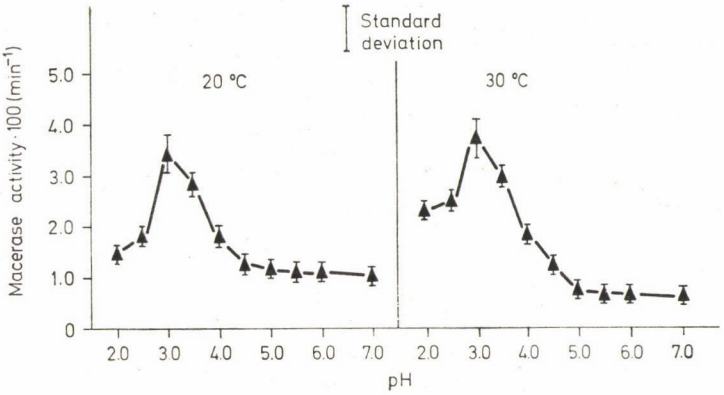

Fig. 3. Effect of pH on the activity of macerase at 20° and 30°C incubation temperatures (5 parallels at each pH value)

on the ordinate. Each point on the figure is the mean of five experimentally obtained results. The vertical bars going through the points represent the standard deviations.

Fig. 3 shows the enzyme activities of samples incubated at 20° and 30 °C, resp., as functions of pH.

It appears from the figure that the best results have been obtained when the samples were treated with a fermentation broth of pH 3.0. There was no significant difference between the enzyme activities measured at pH 3.0 and 3.5 at any of the incubation temperatures. At all other pH values the enzyme activities were very significantly lower than at pH 3.0. At an incubation temperature of 30 °C the difference between the activities at 2.0, 2.5 and 3.0 pH was significant only at the 95% reliability level.

Changes in the enzyme activities of fermentation broth samples incubated at 40 ° and 50 °C and in a pH range between 2.0 and 7.0 are illustrated in Fig. 4.

The figure shows quite clearly that at both temperatures a pH value of 3.0 gave the best results. Mathematical statistical evaluation has indicated the absence of a significant difference in the enzyme activities of samples incubated at 3.0 and 3.5 pH, resp., at the above two temperatures. The results

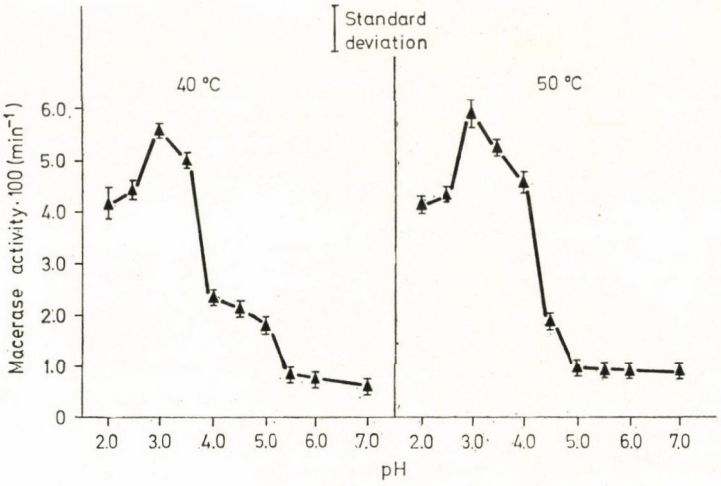

Fig. 4. Effect of pH on the activity of macerase at 40° and 50° incubation temperatures (5 parallels at each pH value)

were significantly lower when the samples were incubated at pH values of 2.0 and 2.5, at both temperatures, or at a pH value of 4.0 at 50 °C, and very significantly lower at any other pH value than the enzyme activities of samples incubated at pH 3.0.

Fig. 5 sums up the results of the determination of the pH optimum for the macerating enzyme at four different temperatures and that of the temperature optimum at pH 3.0.

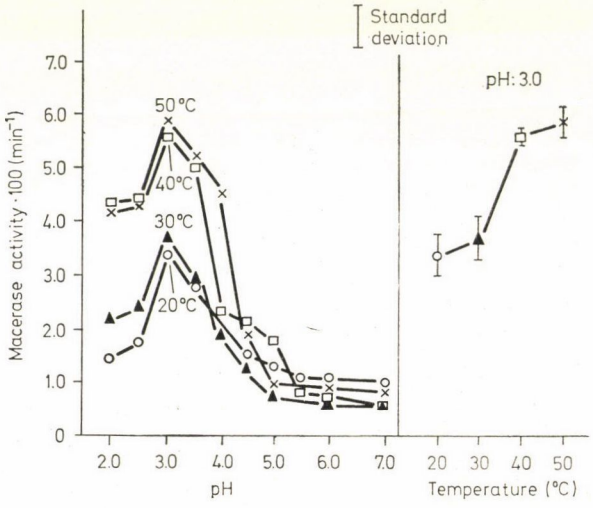

Fig. 5. Determination of the optimum temperature and pH value for macerase enzyme activity (5 parallels at each pH and temperature level)

It can be seen from the figure that at all incubation temperatures the pH optimum is 3.0. If changes in macerase activity are plotted as the function of the incubation temperature (at constant optimum pH value, Fig. 5), it appears that at incubation temperatures of 40 ° and 50 °C the activity of the enzyme is 1.5 and 2.0 times that of the activity measured at 20 ° and 30 °C, resp. Because of the danger of denaturation of the potato tissues, higher temperatures cannot be used.

2.2. Effect of enzyme concentration

The effect of enzyme concentration on the activity of the macerating enzyme was studied partly on the inductive type of polygalacturonase (PG-179) of our own make and on a known preparation of the firm Rohm and Haas (Pectinol R-10).

Of these preparations solutions of 0.1, 1.0, 10 and 20 mg · ml^{-1} enzyme concentration were prepared in the McIlvaine buffer of pH 3.0. The tests were carried out with 5 ml amounts of these solutions. The results are shown in Fig. 6.

It appears quite clearly from the figure that it is not expedient to use enzyme concentrations higher than 5.0 mg · ml^{-1}.

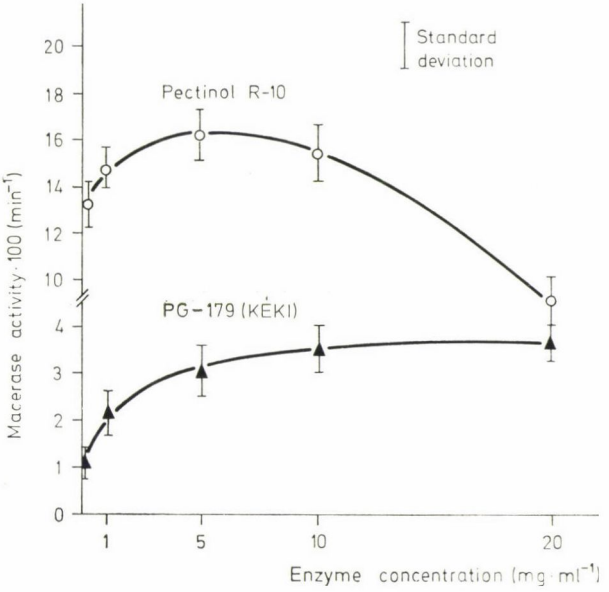

Fig. 6. Effect of enzyme concentration on the activity of the maceration enzyme. Comparison of the macerase enzyme activities of inductive polygalacturonase preparation PG-179 (KÉKI = Central Food Research Institute) and of Pectinol R-10 (Rohm and Haas, Philadelphia, Pennsylvania) pH: 3.0, temperature 30°C, 5 parallel measurements at each concentration level

Some microscopic pictures were taken to show the effect of the macerase enzyme. To improve the picture instead of 1.0 mm now about 0.5 mm thick slices of potato were prepared which were placed on the slides and a few drops of the enzyme solution of 10 $mg \cdot ml^{-1}$ enzyme concentration added, the sample covered with a glass plate and incubated at 30 °C. After an incubation period of 90 minutes the cell walls have completely disappeared from the part of the sample in contact with the solution of the enzyme.

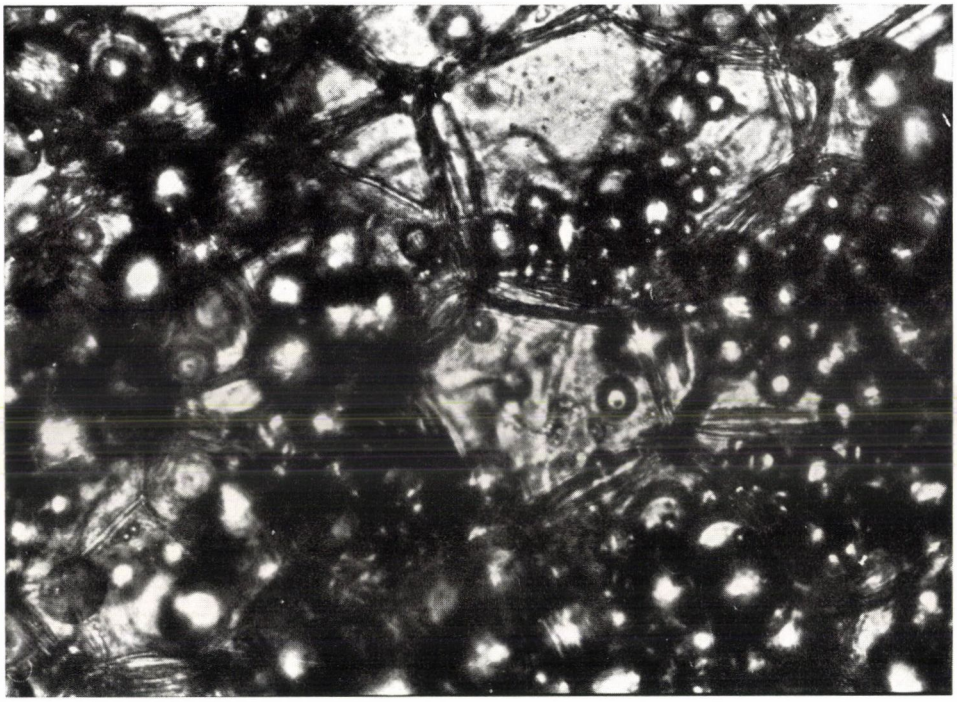

Fig. 7. Potato tissue prior to maceration (Microphotograph, 100× magnification)

Fig. 7 is the microphotograph of the potato tissue prior to maceration (100× magnification), while the macerated tissue is shown in Fig. 8.

The histological structure of the potato tuber consisting of parenchymal cells with starch particles deposited in them is clearly visible in Fig. 7. After the reaction only the starch particles and cell wall fragments left behind after the destruction of the bulk of the cell wall can be seen (Fig. 8). The real effect of maceration, *i.e.* production of single cells after splitting the middle lamella among neighbouring cells cannot be seen in Fig. 8 because of the simultaneous action of other enzymes (cellulase, haemicellulases *etc.*) being present in the preparations.

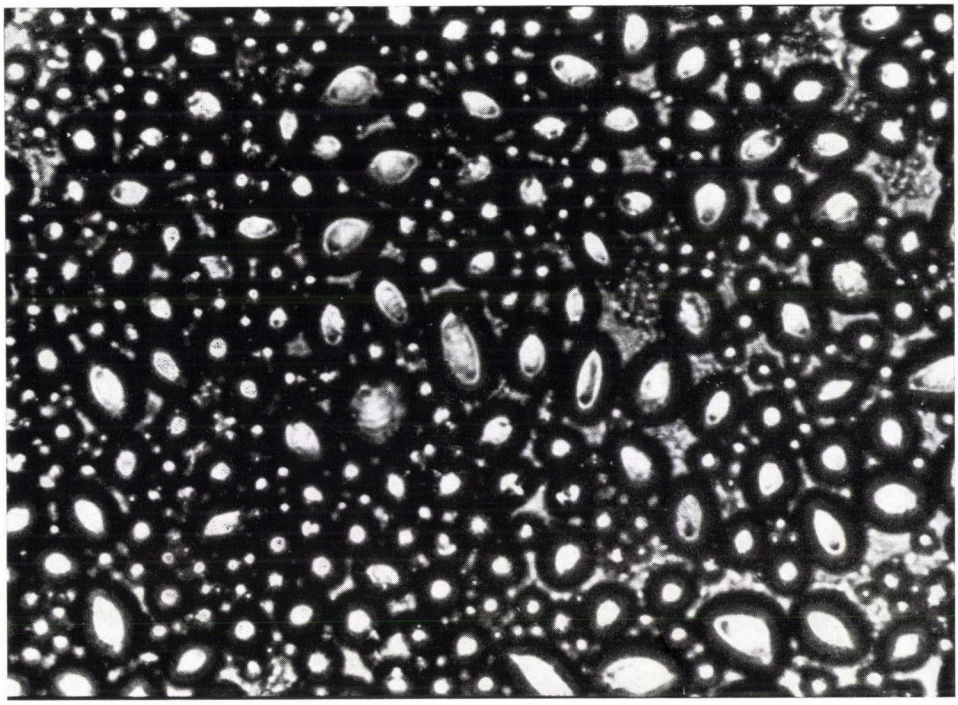

Fig. 8. Potato tissue after enzymatic treatment (Microphotograph, 100× magnification)

3. Conclusions

Macerase is the enzyme which performs the destruction of the middle lamella between the cells of plant tissues. Neither its mechanism of action, nor its composition are accurately known. Its activity can be measured merely on the basis of its effect on native plant tissue substrate. It presumably hydrolyses, among others, the insoluble pectin in the middle lamella of plant cells.

Since the hitherto used subjective methods involve a fairly great possibility of committing errors in the determination of enzyme activity, with the aim of eliminating these errors a simple device was constructed in our Institute by means of which it is possible to follow the collapse of the walls of plant cells in a more exact and less subjective manner.

In the course of our microscopic investigations we were unable to prepare individual cells characteristic of the macerase enzyme, since the hydrolyzing effect of the other enzyme components in the preparation apparently interfered with these effects. It has, however, been established that the method is sufficiently sensitive, well reproducible and suitable for following the destructive maceration of plant tissues, *i.e.* of cell walls.

The two pH optima of macerase observed by Japanese authors (KAJI *et al.*, 1966) was confirmed for Pectinol R-10, but could not be reproduced with our own enzyme preparation (Fig. 9) with the exception perhaps of the pH curve measured at an incubation temperature of 40 °C, where extreme values were observed both at pH 4.0 and 5.0 (Fig. 5). These, however, are the result not of an increase in activity, but only of a more moderate decrease in activity.

The pH value of 3.0, which was found as the optimum pH for macerase, is in agreement with the pH optimum of polygalacturonase. Thus, our measurements confirmed the earlier observation of LUH and PHAFF (1954) and of KAJI and co-workers (1966) according to which endo-polygalacturonase may be responsible for the maceration effect.

The pH optimum observed with our own preparations is in contradiction with statements claiming an identity between macerase and pectin-transeliminase (BYRDE & FIELDING, 1968; ISHII & YOKOTSUKA, 1971). The slightly extreme values at incubations at 40 °C (Fig. 5), agree at pH 4.0 with the pH optimum of xylanase and arabanase, at pH 4.5 with that of apple-juice-clarifying polygalacturonase (ZETELAKI-HORVÁTH, 1972) and at pH 5.0 with that

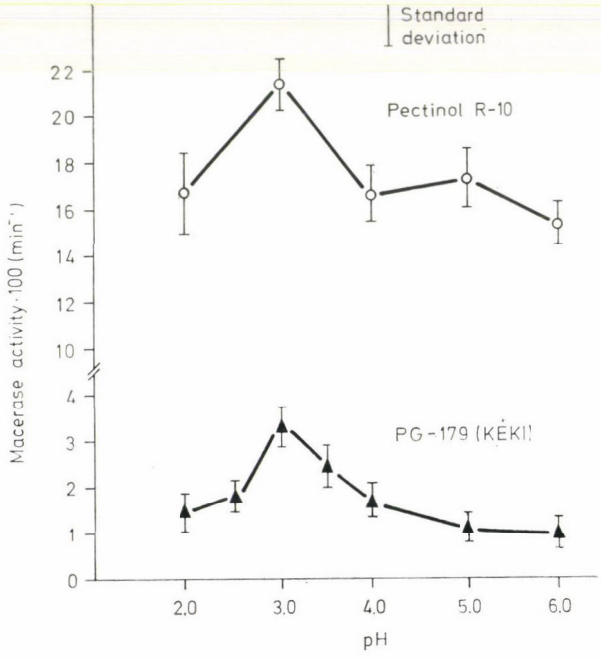

Fig. 9. pH dependence of the maceration effects of Pectinol R-10 (Rohm and Haas, Philadelphia) and of PG-179 (KÉKI = Central Food Research Institute). (Temperature: 30°C, enzyme concentration: 10^3mg · ml^{-1})

of pectin-transeliminase. However, the maceration activities at these pH values are only about one third or less of the activity at pH 3.0.

In the case of Pectinol R-10 the second peak at pH 5.0 indicates a more pronounced effect of pectin-transeliminase, but this value is still very highly significantly ($P \geq 99.9$) lower than the activity maximum at pH 3.0. The second pH optimum of Pectinol R-10 can be more easily attributed to the participation of pectin-transeliminase in maceration, since, in comparison with pH 4.0, a marked activity increase was noted at pH 5.0.

Since the aim of this work was merely to find a suitable test method, it would not be appropriate to draw far-reaching conclusions from these experiments. The discrepancies found between the different enzyme preparations, as well as the contradictory data in the literature indicate differences in the composition of the enzyme preparations, that is in the synthetizing ability of the producing microorganisms. They point to the justified differences among literature data and support the view that further thorough research is needed for the identification of macerase enzyme.

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METHODS FOR MEASURING MACERASE ACTIVITY

PART II. – DETERMINATION OF MACERASE ACTIVITY FROM THE WEIGHT LOSS

K. ZETELAKI-HORVÁTH

(Received May 19, 1973)

An objective method for measuring macerase activity is suggested which is based on the determination of the weight loss due to the enzymatic degradation of plant tissues.

The vegetables (potato, carrot, cucumber) were cut into cylindrical samples of definite dimensions, the samples weighed on the analytical balance and incubated in the enzyme solution under investigation for a definite time. During the period of the reaction the plant cells underwent maceration, so that the outer tissue layers of the cylinders were disintegrated. At the end of the reaction the cylinders were blotted with filter paper to remove the moisture and reweighed with the same accuracy as before. The weight loss of the samples allows conclusions on the macerase activity of the enzyme solution under investigation. By this method four parallels of 10 to 15 samples can be tested daily. The method has the added advantage of not requiring any special instrument or equipment, it can be used wherever test tubes, thermostat and an analytical balance are available.

The determination of macerase activity by the gravimetric method given in this paper is reproducible. The enzyme concentration range appropriate for the investigation of macerase activity by this method was found to be 1.0 and 10mg · $\cdot$ ml^{-1}. The optimum pH of the maceration effect varied with the kinds of substrates used, pH 2.5–3.5 when using potatoes, pH 3.0–4.0 with cucumber and pH 3.0 with carrots. 40°C is recommended as incubation temperature in case of potato and carrot substrates, while 30°C in that of cucumber substrate. The shortest incubation time was found to be necessary with cucumbers (1.5 hour) while in case of potato and carrot substrates 3.0–3.5 hours of incubation were necessary.

From three kinds of plant tissues used as substrates, potato is the least sensitive but has most of the advantages, like easy handling, uniform texture and availability all over the year.

The two other substrates, although more sensitive to tissue disintegration, appear to be less suitable for measuring macerase activity. The lack of a constant ratio of bast tissue to trunk in carrot substrate is a limiting factor of its application. Cucumber cannot be recommended because it is not available all year round and because of the high standard deviation of the results obtained.

The phytopathologists WOOD (1955) and BROWN (1965) were the first to call attention to the existence of a macerating enzyme (macerase). This was soon followed by the publication of several papers discussing the problems of the action and identification of this enzyme (KAJI & ANABUKI, 1956; MCCLENDON, 1964; BUSH & CODNER, 1968).

The effect of macerase, mainly the destruction of the middle lamella between the cells of plant tissues by the enzyme, has an important bearing on the possibilities of its practical application. In the fruit juice industry it enables the production of homogeneous fibrous juices. Preliminary degradation

of green vegetables might be an important application in the canning industry. When using the enzyme in histological and phyto-anatomical tests it is possible to subject the plant tissues to an aimed and mild decomposition.

If the enzyme is to be used in practice, a simple activity test is required to determine the dose needed for treatment and to check enzymatic effect. In Part I of this work we have described an instrumental method for measuring the activity of the macerating enzyme (ZETELAKI-HORVÁTH, 1974), now we wish to report on a method based on the gravimetric determination of the weight loss occurring as a result of the enzymatic maceration of the tissue. Our objective was to work out a rapid, simple and easily reproducible method which requires no particular equipment.

By measuring macerase activity as the incurred weight loss, the incubation period of the enzyme reaction, as well as its optimum pH and temperature conditions were determined using potato, carrot and cucumber tissue cylinders as substrates. The enzyme concentration which lends itself to the test was also determined.

1. Materials and methods

1.1. Method

For the gravimetric determination of the enzyme activity of macerase, potato, carrot and cucumber tissues were used as substrates. With the help of the cork-borer and a two-blade cutting device cylinders of 10 mm diameter, 20 mm height and approx. 1.0 g weight were cut from the vegetables. In the case of carrot the cylinders contained both the bast tissue and the core consisting of ligno-parenchyma cells. The potato specimen consisted of the parenchyma cells of the flesh, while the mesocarpium made up the substrate tissue system of the cucumber sample.

The pH values of the fermentation broth samples under investigation were adjusted to the desired level by the addition of hydrochloric acid or of caustic soda, as required. Fifteen millilitres of the culture broth were pipetted into test tubes, the cylinders made of the plant tissues placed into the broth and incubated at the desired temperature for a given period of time. Fermentation broths of the same pH values and containing thermally inactivated enzyme were used as controls.

At the end of the incubation period the moisture on the cylinders was removed by means of blotting paper and the samples reweighed on the analytical balance up to the nearest mg. Weight losses were expressed as percentage of the initial weight. Weight losses of the control samples are also shown in the figures with dotted lines. Each experiment was performed in four parallels on substrates of the same type and from the same place.

1.2. Mathematical statistical methods

The standard deviations of results obtained by using different parameters were calculated and the effect of the parameters compared by means of the t test (WEBER, 1972).

1.3. The enzyme preparations

PG-C_1 (Central Food Research Institute, pilot production) is an inductive type liquid polygalacturonase preparation with macerating effect, produced by an *Aspergillus foetidus* strain (ZETELAKI & VAS, 1972). The liquid preparation contained the following polygalacturonase components: endo-polygalacturonase (endo-PG) $SPA_{75}^{Na\text{-}pectate}$: 1 670 $l \cdot h^{-1} \cdot l^{-1}$ (determined on sodium polypectate substrate); endo-polymethylgalacturonase (endo-PMG) SPA_{75}^{Pom}: 327 $l \cdot h^{-1} \cdot l^{-1}$ (determined on Pomosin pectin substrate, degree of esterification: 70%) and apple-juice-clarifying polygalacturonase, SPA_{75}^{A}: 1 800 $l \cdot h^{-1} \cdot l^{-1}$, determined on apple-juice (Jonathan variety) of an initial specific viscosity of 1.0 and a pH value of 3.8.

The polygalacturonase powder, No. PG-179 was prepared from the culture filtrate of submerged *Aspergillus foetidus* fermentation by precipitation with methanol (experimental production, Central Food Research Institute). The enzyme concentrations were: endo-polygalacturonase, $SPA_{75}^{Na\text{-}pectate}$: 94 200 $l \cdot h^{-1} \cdot kg^{-1}$, endo-polymethylgalacturonase, SPA_{75}^{Pom}: 22 600 $l \cdot h^{-1} \cdot kg^{-1}$, apple-juice-clarifying polygalacturonase, SPA_{75}^{A}: 288 000 $l \cdot h^{-1} \cdot kg^{-1}$.

1.4. Determination of enzymatic activity

The polygalacturonase activities (endo-PG, endo-PMG, apple-juice-clarifying PG) of the enzyme preparations were determined from the viscosity decrease of the substrate (ZETELAKI & VAS, 1972).

The following tests carried out using various plant tissues as substrates were to determine the activity of macerating enzyme.

2. Results

2.1. The effect of pH and temperature on macerase activity in case of potato tissue substrate

Potatoes of the Hungarian variety *Gülbaba* were used as substrates. The effect of the two parameters on the enzymatic activity of macerase was tested for each value in four parallel experiments.

To establish the optimum pH for enzyme activity, experiments were performed in the pH range between 2.0 and 7.0 at intervals of 0.5 pH unit.

The incubation temperatures were 20°, 30°, 40° and 50 °C, the effect of the length of the incubation period was tested every hour during the five-hour incubation period.

Fig. 1 shows the weight loss of the cylindrical potato samples caused by the effect of macerase, as a function of the pH, temperature and incubation

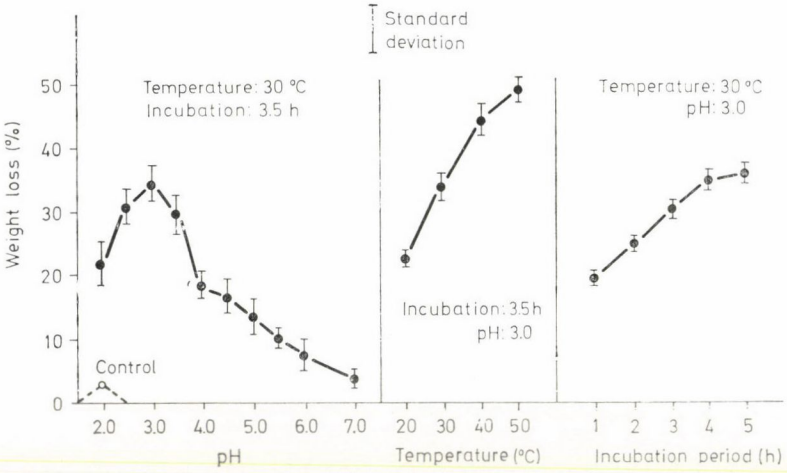

Fig. 1. Weight loss of potato cylinders caused by the action of macerase as a function of pH, incubation temperature and the length of the incubation period, resp. (Enzyme preparation: PG—C_1, KÉKI*)

* KÉKI = Central Food Research Institute

period, resp. The points in the figure represent the means of four parallel experiments.

It appears in the figure that enzyme activity increases with increasing incubation periods (from 1 to 4 hours) and at higher temperatures (between 20° to 50 °C). A temperature of 50 °C was found to be too high if incubation lasted for several hours, causing a change in the texture of those so that this incubation temperature is not recommended, as a rule, when this method is employed.

The effect of pH was tested at 30 °C with an incubation period of 3.5 hours. It can be seen in the figure that from the aspect of enzymatic activity pH 3.0 is the optimum value. There was no significant difference between the weight losses measured at 2.5, 3.0 and 3.5 pH, but the results at lower pH values were very highly significantly lower than those obtained at pH 3.0. Of the control samples only the one at pH 2.0 and containing inactivated enzyme suffered a certain weight loss, all potato cylinders treated with fermentation liquors of other pH values gained in weight. The highest weight

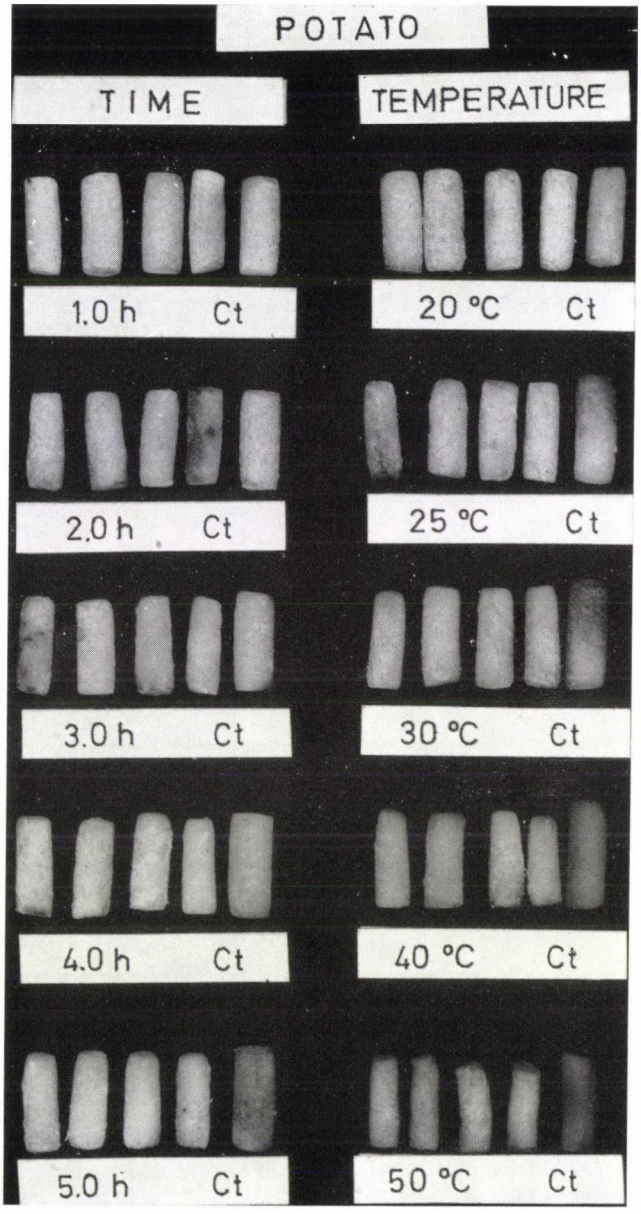

Fig. 2. Morphological changes of potato cylinders after enzymatic treatments of various length (pH 3.0, incubation temperature 30°C) and at various incubation temperatures (incubation period 3.5 hours, pH 3.0, Ct = control sample; enzyme preparation: PG – C_1, KÉKI)

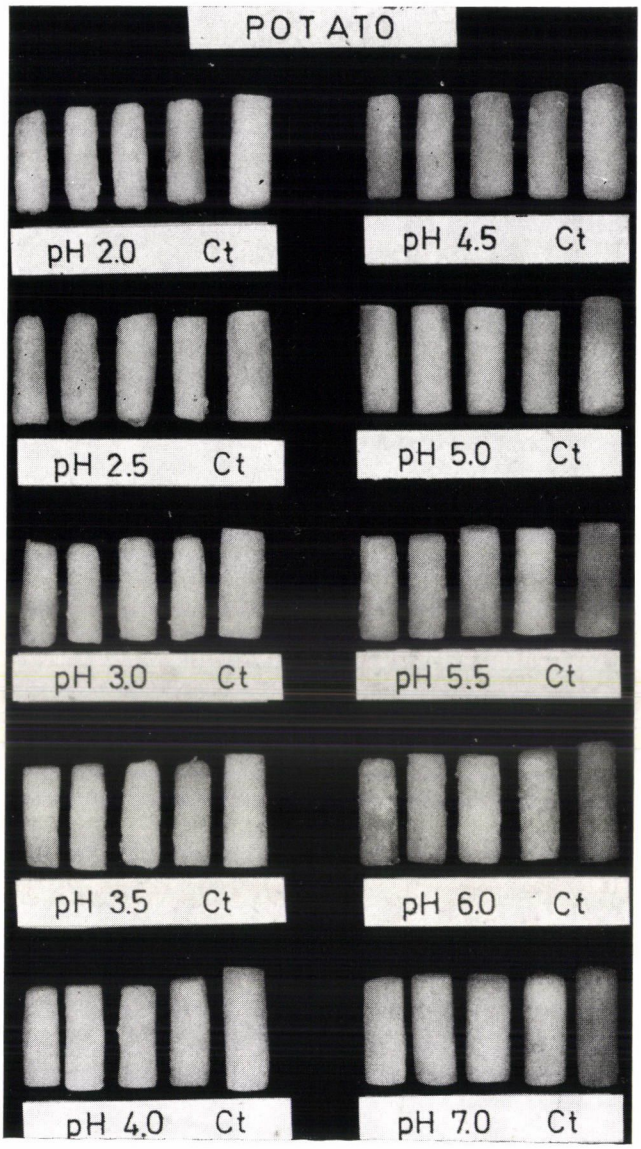

Fig. 3. Morphological changes of potato cylinders after enzymatic treatment in broths of various pH values (length of the incubation period 3.5 hours, temperature 30°C, Ct = control sample; enzyme preparation: PG-C_1, KÉKI)

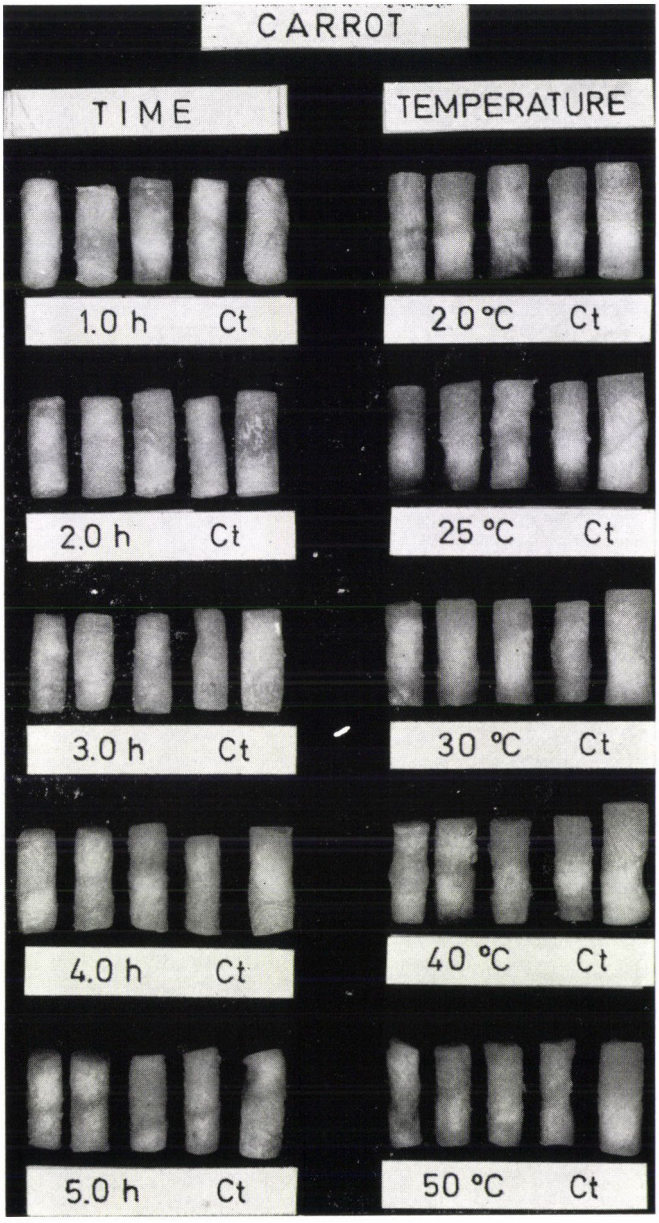

Fig. 5. Morphological changes of carrot cylinders after enzymatic treatments of various length (pH 3.0, incubation temperature 30°C) and at various incubation temperatures (incubation period 3.5 hours, pH 3.0, Ct = control sample; enzyme preparation: PG-C_1, KÉKI)

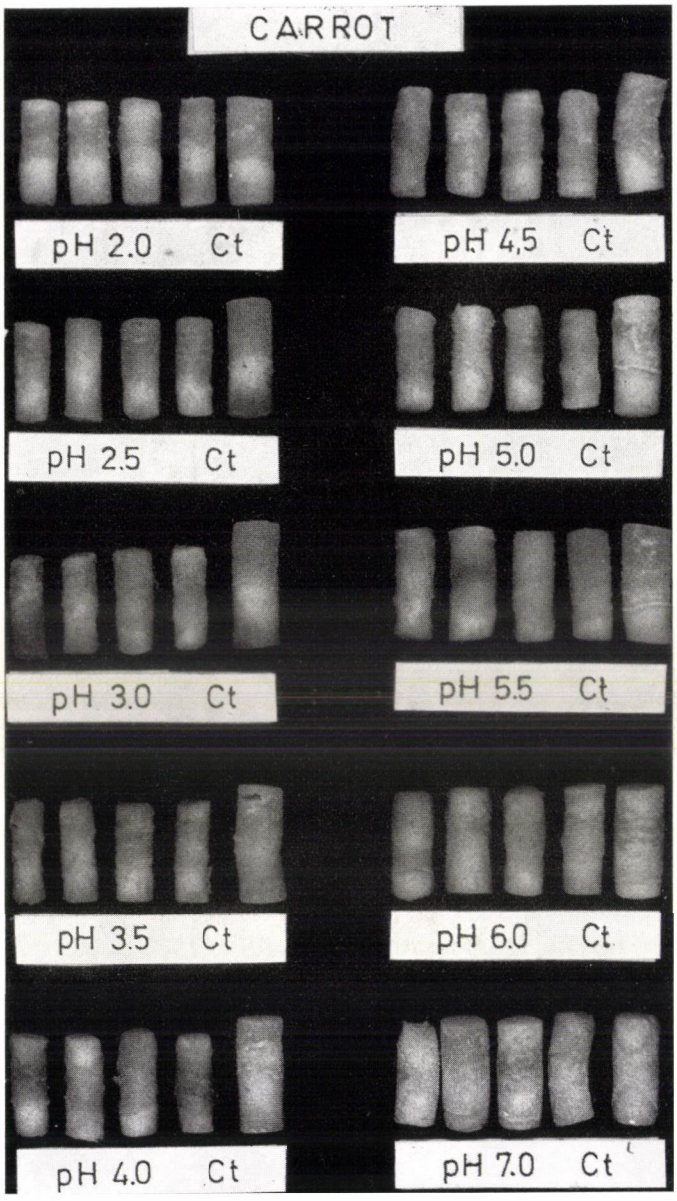

Fig. 6. Morphological changes of carrot cylinders after enzymatic treatment in liquors of various pH values (incubation period 3.5 hours, temperature 30°C, Ct = control sample; enzyme preparation: PG-C_1, KÉKI)

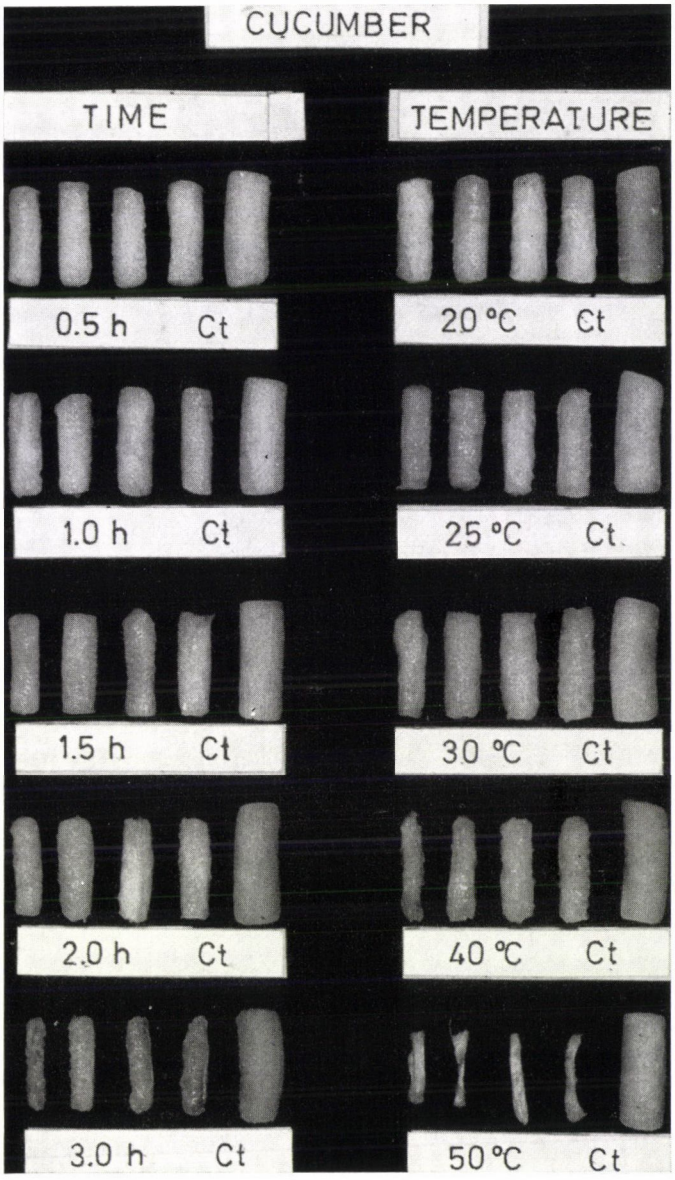

Fig. 8. Morphological changes of cucumber cylinders after enzymatic treatments of various length (pH 3.0, temperature 40°C) and at various incubation temperatures (incubation period 90 minutes, pH 3.0, Ct = control sample; enzyme preparation: PG-C_1, KÉKI)

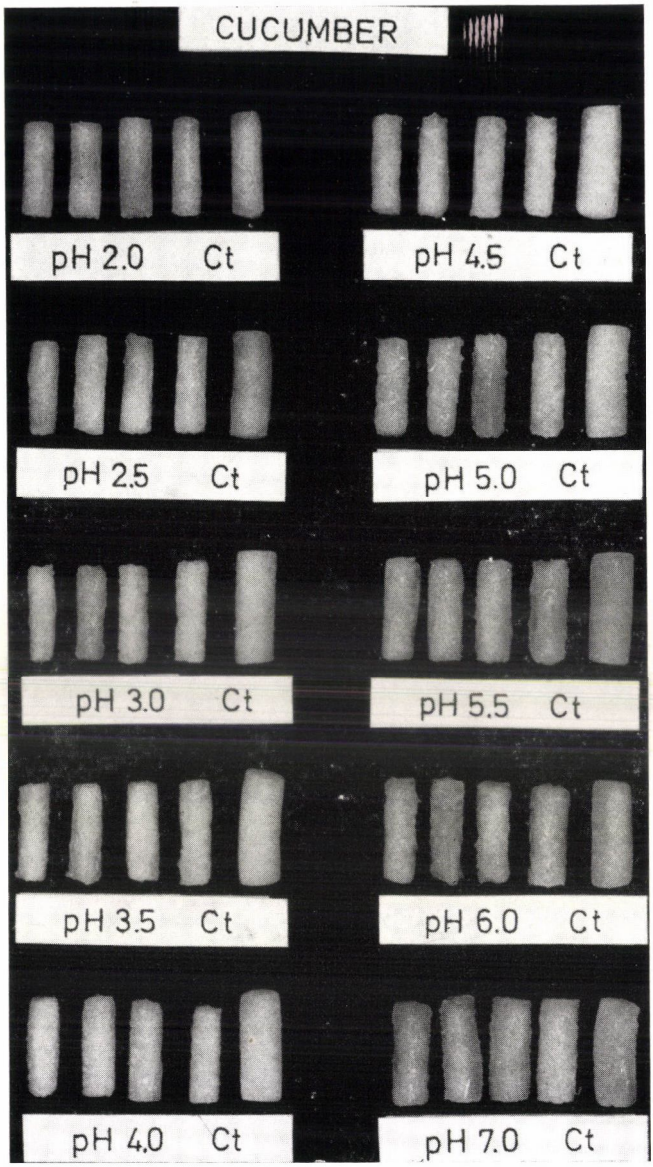

Fig. 9. Morphological changes of cucumber cylinders after enzymatic treatment in liquors of various pH values (incubation period 90 minutes, temperature 40°C, Ct = control sample; enzyme preparation: PG-C_1, KÉKI)

loss in case of potato substrate was 34 to 35% at 30 °C, pH 3.0 and an incubation period of 3.5 hours.

Morphological changes due to the enzymatic treatment of the potato cylinder are shown in Figs. 2 and 3.

It appears further from Fig. 2 that as temperature and length of incubation are raised, the dimensions of the cylinder decrease. The dimensions of the control samples *Ct* are considerably greater than the dimensions of the other cylinders, showing that no weight loss has occurred.

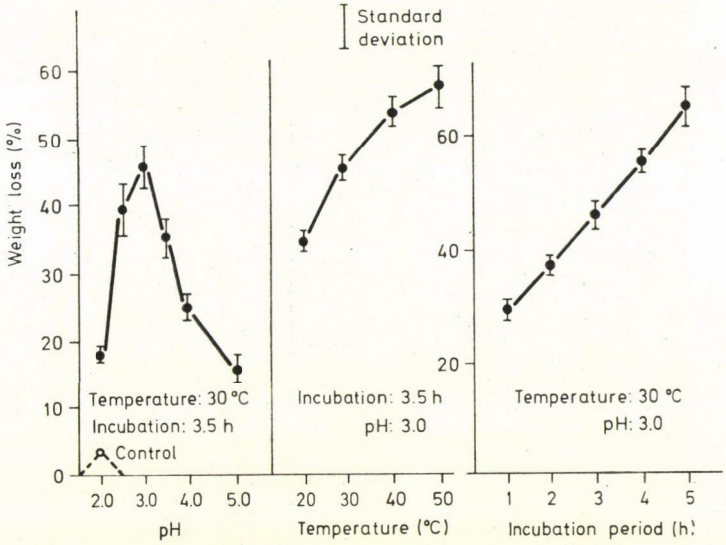

Fig. 4. Weight loss of carrot cylinders caused by the action of macerase as a function of pH, incubation temperature and the length of the incubation period, resp. (Enzyme preparation: PG-C_1, KÉKI)

2.2. The effect of pH and temperature on macerase activity in case of carrot tissue substrate

The optimum pH, temperature and length of incubation were determined with the above enzyme preparations using carrot as a substrate. The results are shown in Fig. 4.

In case of carrot substrate weight loss again increased as the incubation period and temperature were raised. Investigation of the effect of pH in the range between 2.0 and 7.0 showed an optimum maceration effect at pH 3.0 also in the case of carrot. At an incubation temperature of 30 °C the effect was significantly better at pH 3.0 than at pH 3.5 and highly significantly better than at pH 2.0 and 4.0. There was no significant difference between the effects at pH 2.5 and at 3.0. The carrot tissue incurred a 45% weight loss as a result of the enzymatic effect.

Morphological changes of the carrot cylinders after enzymatic treatment are shown in Figs. 5 and 6.

It is clearly visible in Fig. 5 that with increasing temperature and incubation period the weight losses of the carrot tissues have risen.

Fig. 6. shows that samples incubated at pH 3.0 and 3.5 suffered the greatest weight loss, while in the pH range between 4.5 and 7.0 there is practically no weight loss. With the exception of pH 2.0 the control samples gained in weight.

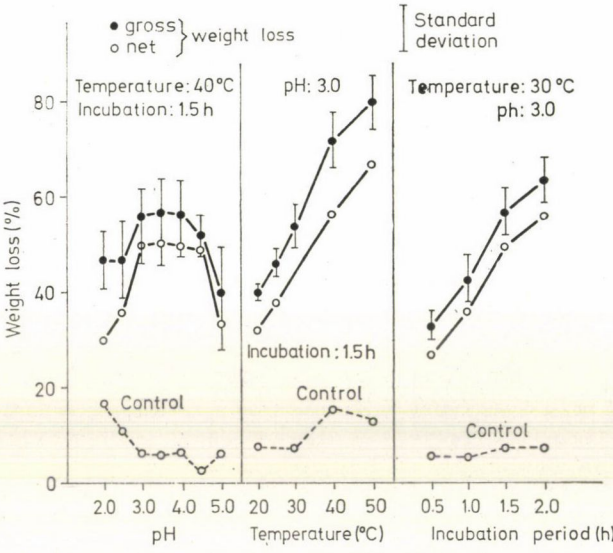

Fig. 7. Weight loss of cucumber cylinders caused by the action of macerase as a function of pH, incubation temperature and length of the incubation period (enzyme preparation: PG-C_1, KÉKI)

2.3. The effect of pH and temperature on macerase activity in case of cucumber tissue substrate

The third investigated plant tissue was the mesocarpium of cucumber. In this case the effect of incubation temperature and time was tested in the temperature range between 20° and 50 °C in the period between 30 minutes and two hours, while the effect of pH was studied in the range between pH 2.0 and 7.0. The results are summarised in Fig. 7. The figure shows the weight losses of the cucumber tissue *vs.* pH, temperature and incubation period, resp. Since in the case of cucumber the control samples displayed a weight loss at all tested pH values, the figure shows in addition to the gross and control weight losses also the net weight loss.

It appears from the figure that the incubation period was shorter than that of the previous substrates. When the period of incubation was raised from 30 to 90 minutes there was an increase in the weight loss, but a further

prolongation of the incubation period had no significant influence on weight loss.

Higher temperatures caused also in the case of cucumber an enhancement of the maceration effect. At 50 °C the weight loss was already so great that it was difficult to reweigh accurately the macerated tissues after 1.5 hours of incubation.

The effect of pH was investigated in the range of 2.0 to 7.0 pH. Here again pH values between 3.0 and 4.0 were found to be the best for the enzymatic maceration of cucumber tissues. There was no significant difference between the maceration effects at pH 3.0, 4.0 and 4.5, while the results obtained at pH 2.0, 2.5 and 5.0, resp., were highly significantly ($P \geq 99\%$) lower than the weight loss measured at pH 3.0.

The morphological changes suffered by cucumber tissues as a result of enzymatic treatment are shown in Figs. 8 and 9.

It appears from Fig. 8 that a prolongation of the incubation period caused a decrease in the weight of the cucumber cylinders. Weight loss incurred by a 3-hour incubation period is so great that it will cause difficulties in the accurate determination of the weight. Higher incubation temperatures, too, will considerably enhance the activity of the macerase enzyme. At temperatures above 30 °C maceration will surpass the desirable limit.

3. Conclusions

Our experimental results demonstrate the suitability of the described method for the detection of macerase activity.

Cucumber was used by MUSSEL and MORRE (1969) as a substrate for the rapid determination of the enzyme polygalacturonase.

In our work concerned with the determination of macerase activity cucumber was found to be the most sensitive substrate. Cucumber cylinders suffered a gross weight loss of approx. 60% when subjected to the action of the enzyme at a temperature of 30 °C for 1.5 hours. After subtraction of the weight loss of the control sample the net weight loss due to enzyme action is about 50% (Fig. 7).

The next most sensitive substrate is carrot with about 45% net weight loss, though this weight loss was only reached during a longer — almost twofold — incubation period than the one measured in the case of cucumber.

Potato as a substrate lost not more than 35% of its weight when incubated at 30 °C for 3.5 hours (net loss).

The three types of substrates were compared by using a polygalacturonase powder preparation (PG-179, KÉKI*) at the optimum pH value of 3.0 and the optimum length of incubation for each substrate (Table 1). A temperature of 40 °C was found to be more expedient causing higher net weight losses of all

three substrates (43.4% for potato, 52% for carrot and 59% for cucumber) than 30 °C.

Table 1

Comparison of macerase activity on three vegetable tissue substrates at optimum pH values

Enzyme preparation: PG-179 (see para. 1.3.)

Average weight of substrate (g)	pH	Temperature (°C)	Incubation period (h)	Average weight loss		DF	Standard deviation (%)	Significance of difference of results at pH 3.0 and pH			
				(g)	(%)			2.5	3.5	4.0	4.5
Potato											
1.0243	3.0	30	3.5	0.300	29.3	8	3.23	—	—	***	***
1.0923	3.0	40	3.5	4.448	41.0	8	4.46	—	—	***	***
Carrot											
0.9362	3.0	30	3.5	0.416	44.4	8	5.42	—	*	**	***
0.9817	3.0	40	3.5	0.510	52.0	8	6.47	—	*	**	***
Cucumber											
0.8872	3.0	30	1.5	0.4527	51.0	8	7.23	—	—	—	**
0.8950	3.0	40	1.5	0.531	59.3	8	12.00	—	—	—	**

— not significant
* significant $P \geq 95\%$
** highly significant $P \geq 99\%$
*** very highly significant $P \geq 99.9\%$

At both temperatures the results of the cucumber substrate showed the greatest and those of the potato substrate the least scattering. There were no significant differences between the results obtained at the optimum pH value of 3.0 and those found at 2.5 and 3.5 in case of potato substrates, or at 2.5, 3.5 and 4.5, resp. with cucumber and 2.5 pH with carrot substrate. pH values above 4.0 gave for all three substrates (even pH 4.0 in case of potato) highly significantly lower ($P \geq 99.9\%$) macerating activities than pH 3.0. In case of carrot pH 3.5 reduced significantly, pH 4.0 highly significantly the maceration activity of the preparation compared to the activity at pH 3.0.

The enzyme concentration range appropriate for the investigation of macerase activity by means of weight loss determination was found to be between 1 and 10 $mg \cdot ml^{-1}$ using the preparation PG-179 with potato tissue substrate (Fig. 10).

Comparison of the three substrates showed that potato tissue is the least sensitive, but has the advantage of easy handling, uniform texture, the lowest standard deviation values and mainly the fact that the same variety is always available irrespective of the season.

The difficulty to ensure identical ratios between the bast tissue and the trunk consisting of wood parenchyma cells in the parallel cylinders used in the experiments is a limiting factor in the use of carrot as substrate, since the lack of a constant composition might introduce considerable discrepancies among the results of routine tests.

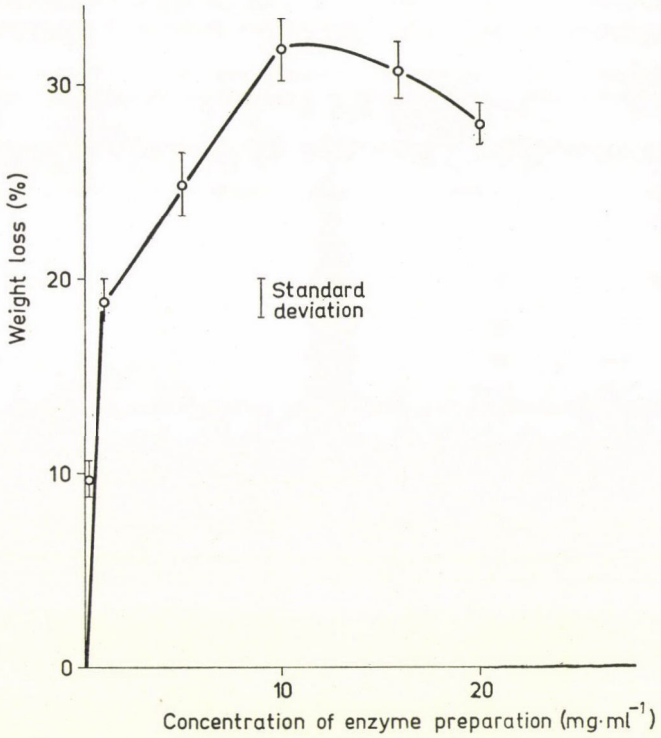

Fig. 10. Effect of enzyme concentration on the activity of the maceration enzyme. (Enzyme preparation PG-179, KÉKI, see para. 1.3., substrate: potato tissue, pH 3.0, incubation period 3.5 hours)

Since cucumber is not available all year round, its application has certain drawbacks, though it is planned to study the applicability of deep frozen cucumber.

The pH optimum of the maceration effect was found in case of potato and cucumber substrates in the range between 2.5 and 3.5, and 3.0 and 4.0 pH resp., while for carrot substrate the highest macerase activity was measured at pH 3.0. At neutral or near neutral pH values the tissues absorbed water and thus their weight increased and no maceration could be observed.

The recommended temperature for incubation is 30° or 40 °C, since at higher temperatures undesirable changes might occur in the initial properties of native tissues.

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ESTIMATION OF VOLATILE ACIDS IN EMMENTAL CHEESE BY GAS-LIQUID CHROMATOGRAPHY

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A method has been developed for gas chromatographic separation and quantitative estimation of volatile acids of Emmental cheese.

Volatile acids were extracted from cheese by steam distillation, concentrated to dryness as Na salts and subsequently set free by concentrated H_2SO_4 under cooling. The free acids were dissolved in diethyl ether, residual water was eliminated by anhydrous Na_2SO_4 and, finally, ether was evaporated at 30°C on a water bath.

Chromatographic separation was performed in a Perkin-Elmer 900 gas chromatograph equipped with a flame ionisation detector. Dimethyl chlorosilane treated Chromosorb W was used as carrier with 10% polyethylene glycol adipate as stationary phase, and hydrogen as carrier gas. Identification was carried out on the basis of retention distances by comparison with the chromatogram of a standard mixture and relative amounts of individual acids were calculated from peak areas. The concentrations of the acids in cheese were calculated by relating their relative amounts to total free volatile acidity as determined by titration of the steam distillate.

It was established that under identical manufacturing conditions the clotting enzyme had a marked influence on volatile acid composition of the product: in Emmental cheese produced with a microbial preparation from *Endothia parasitica* propionic acid content was considerably higher as compared to cheese obtained with calf rennet, while butyric acid was formed to a lesser degree or not at all. Total free volatile acidity was also significantly higher in cheese manufactured with the microbial preparations than in the batches produced in parallel with calf rennet.

Short chain volatile carboxylic acids are known to play an important role in cheese flavour formation. While KEVEI and co-workers (1968, 1969) were concerned with the determination by gas-liquid chromatography of higher fatty acids in foods, this paper deals with a combined titrimetric — gas chromatographic method that has been developed for the estimation of fatty acids up to C_{10}. Cheese batches manufactured with calf stomach rennet and with a microbial milk clotting preparation obtained in this laboratory by submerged fermentation of an *Endothia parasitica* mould strain were compared with respect to total free volatile acid content as well as volatile acid distribution.

1. Materials and methods

1.1. Cheese samples

Two batches each of Swiss type "Emmental" cheese produced with calf rennet and microbial rennet in powdered and liquid form, respectively,

were tested for their C_2-C_8 carboxylic acid composition as well as total free volatile acid (VA) content after a ripening period of 3 months. One of the cheese samples manufactured with the powdered microbial enzyme preparation was tested also after two months of ripening. (The same equalized milk was used for manufacturing cheese in parallel with the two types of enzymes.) Since all the cheese samples investigated had the same moisture content results were related to the weight of cheese and not to solids.

Emmental cheese purchased from Budapest retail shops was partly used to establish the conditions of volatile acid isolation.

1.2. Determination of total free volatile acid content

Volatile acids were extracted from cheese by steam distillation. 50 g of freshly grated cheese and 50 ml of boiled distilled water were weighed into a 1 000 ml Kjeldahl flask with a neck of 3 cm length. The distillate was collected into a known excess (20—50 ml) of carbonate-free 0.1 *N* NaOH. In order to establish the amount of distillate necessary to recover total free volatile acid content 120 ml fractions were collected, 50 ml aliquots of which were titrated with 0.01 *N* HCl in the presence of phenolphthalein. Volatile acid content in the fractions was calculated from titration results and expressed as mg acetic acid and meq, respectively.

Distillation was carried on till the fractions were practically free of acid. The acid containing fractions were combined and treated as described in para. 1. 3.

1.3. Preparation of the samples for gas-liquid chromatography

A 50 ml aliquot of the distillate containing the volatile acids was used to determine VA content of cheese by titration with 0.01 *N* HCl, according to para. 1.2. The rest of the distillate was concentrated to dryness under vacuum at 100 °C. After cooling to 5—10 °C the dry mixture of the sodium salts of the acids and of excess NaOH was overlayered by 30 ml of diethyl ether. The vessel containing the mixture was then positioned in a bath of melting ice and 1 or 2 ml cc. H_2SO_4 were added to its contents to liberate the volatile acids. The mixture was then shaken three times in a separating funnel with 30-ml portions of ethyl ether, the ether layers were combined and allowed to stand over anhydrous Na_2SO_4 in a covered beaker for about 30 min to eliminate traces of water. Subsequently the solution was filtered and ethyl ether evaporated on a 30 °C water bath. After redissolving the residue in 30 ml ethyl ether and repeated treatment with sodium sulfate, followed by filtration and evaporation, the sample was ready to be chromatographed.

1.4. Gas chromatographic separation

A Perkin Elmer model 900 gas chromatograph equipped with a flame ionisation detector was used. The stainless steel columns of 3.6 m length and 2 mm inner diameter were filled with 10—12 g of dimethyl chlorosilane treated 80—100 mesh Chromosorb W containing 10% of polyethylene glycol adipate as stationary phase.

Separation was carried out under isothermal conditions at 180 °C with a carrier gas (N_2) flow of 60 ml·min^{-1}. Attenuation was 4-fold, the amplifier range 100-fold and the rate of paper displacement 3.3 mm·min^{-1}.

Samples containing cheese VA were prepared in triplicate and three parallel gas chromatographic separations were carried out with every sample (by injecting 1 μl). Thus 9 values were obtained for every cheese investigated, the means of which were calculated along with standard deviations.

1.5. Identification of volatile acids

The separated acids were identified on the basis of the linear relationship between the logarithm of the number of carbon atoms of the acids and retention distances by comparing the chromatograms of cheese extracts with those

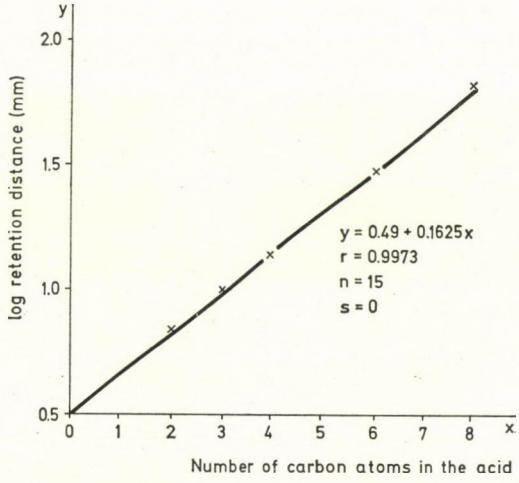

Fig. 1. Relationship between retention distances and number of carbon atoms of volatile acids. Conditions: standard solution: 5 μl each of individual standard acids dissolved in a total of 2.0 ml of diethyl ether; 5 μl of the solution injected into the chromatograph. — Gas chromatography: Perkin Elmer 900, flame ionisation detector; column length 3.6 m, inner ∅ 2 mm; support: 10—12 g 80—100 mesh Chromosorb W treated with dimethyl chlorosilane; stationary phase: 10% polyethylene glycol adipate; injector temperature 100°C, column temperature 180°C, detector temperature 220°C; carrier gas flow 60 ml·min^{-1}, attenuation 4-fold, amplifier range 100-fold, paper displacement rate 3.3 mm·min^{-1}·r = correlation coefficient of regression equation; n = number of measurements; s = standard deviation

of corresponding standard solutions (TRANCHANT, 1969). The mixture of standard volatile acids was prepared by dissolving 5 μl each in a total of 2.0 ml of diethyl ether. 5 μl of this solution were injected into the chromatograph. Fig. 1 illustrates the linear plot as obtained on chromatographing the above mixture.

1.6. Quantitative estimation of volatile acids

Distribution of volatile acids was calculated from the areas under the chromatographic peaks and expressed as percentage of the total area. The concentrations of the individual acids were obtained by relating their percentage values to total free volatile acid content as determined according to para. 1.2. and were expressed in mg per 100 g cheese.

The results were evaluated by analysis of variance and Student's t test.

2. Results

2.1. Extraction of free volatile acids from cheese

VA content of a 50-g sample of commercial Emmental cheese could be completely recovered in 840 ml of distillate, as proven by fractionated steam distillation (Fig. 2).

As can be seen, the first fraction contained less acid than the second, probably owing to the fact that during the first phases of distillation the temperature of the cheese – water mixture was lower than desirable. (Preheating the cheese sample would have caused losses in VA content.) The acid content of the individual fractions as expressed in percentage of total recovery (obtained in 840 ml) is represented in Fig. 3.

The first 420 ml of distillate contain about 85% of the total volatile acids, while another 420 ml are necessary to extract the remaining 15%. Since steam distillation is rather time consuming (2.5—3 h are necessary to collect 420 ml of distillate), collecting an 8-fold volume as related to cheese weight was considered sufficient for further work. Comparison by Student's t test showed that the variations caused by this simplification were within the limits of error with respect to VA content as well as to percentage values of individual acids. These findings hold for commercial Emmental samples with a VA content below 100 mg % (1.7 meq in 100 g). With cheese containing more than 1.7 meq in 100 g of VA a similar performance could be achieved, however, only by collecting a 20-fold volume (*i.e.* 1 000 ml of distillate from a sample of 50 g).

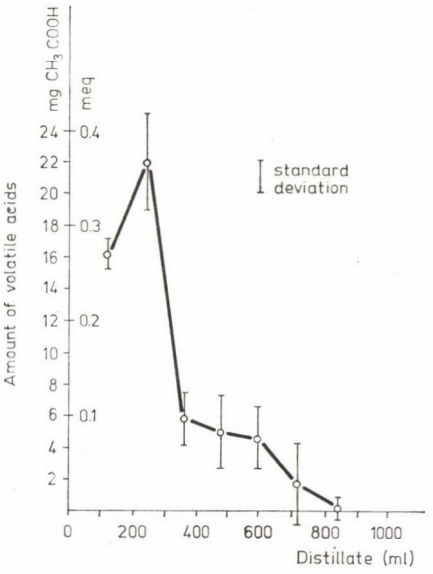

Fig. 2. Relationship between the volume distilled and the volatile acids collected on steam distillation of Emmental cheese. Conditions: 50 g of freshly grated commercial cheese + 50 ml boiled distilled water. Steam distillate collected into 20 – 50 ml of 0.1 *N* NaOH. Determination of volatile acids by titration of excess NaOH with 0.01 *N* HCl

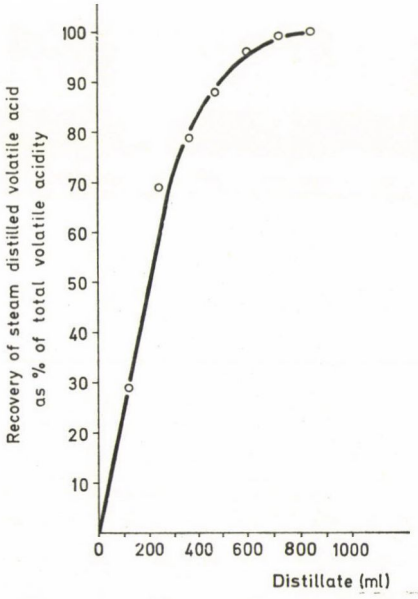

Fig. 3. Relationship between recovery of volatile acids and volume of steam distillate. Conditions as in Fig. 2

These results are illustrated in Table 1.

Table 1

Comparison by Student's t test of the distribution of C_2—C_8 fatty

Cheese samples: *No. 1:* experimental cheese prepared with powdered microbial (108.7 mg CH_3COOH) in 100 g; *No. 2:* commercial cheese; total free volatile acid content:

Sample No.	Volume of steam distillate		Free volatile acids in the distillate, % of total free volatile acidity	Distribution of C_2—C_8			
				C_2		C_3	
	ml	related to sample weight		$\bar{x}$	s	$\bar{x}$	s
1	400 1 000	8 20	90.8 100.0	77.7 78.1	6.8 3.7	22.2 21.9	6.8 3.7
Actual values of t	—	—	—	0.15		0.57	
2	800 1 100	16 22	85.3 100.0	36 39	4.2 9.2	4 4	0.8 0.0
Actual values of t	—	—	—	1.35		0	

$\bar{x}$ = mean value
s = standard deviation

The values of VA content of the cheese samples investigated are represented in Fig. 4.

It can be seen that the samples of the two cheese batches manufactured with calf rennet and ripened during 3 months have similar free VA contents, the values of which do not differ significantly. Free VA content of the sample taken after 2 months of ripening from cheese manufactured with the powdered microbial preparation was of the same order. After 3 months of ripening cheese produced with microbial rennet had a significantly higher free volatile acid content than the respective control samples prepared with calf rennet. The difference is particularly considerable with batch No. 2: free VA content in cheese prepared with the powdered microbial enzyme amounts to nearly the 5-fold, in cheese prepared with the liquid preparation to more than the 3-fold as compared to the value obtained for cheese manufactured with calf rennet.

2.2. Gas chromatographic analysis

The chromatograms of the volatile acids of cheese from batch No. 1 manufactured with calf rennet and microbial rennet, respectively, are represented in Figs. 5 and 6.

Both cheeses contained acetic, propionic, butyric, caproic and caprylic acids, acetic acid being present in the largest amounts. In cheese manufactured

acids in different volumes of steam distillate from cheese

rennet and tested after 2 months of ripening; total free volatile acid content: 1.81 meq 4.13 meq (247.8 mg CH_3COOH) in 100 g. Sample size: 50 g

volatile acids						n	DF	Critical value of t (P = 5%)
C_4		C_6		C_8				
$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s			
0	—	0	—	0	—	9	16	2.12
0	—	0	—	0	—	9		
—		—		—		—	—	—
30	3.1	18	2.6	12	1.7	10	11	2.20
28	4.2	17	4.5	12	4.0	3		
2.01		0.93		0		—	—	—

n = number of parallel determinations
DF = degree of freedom

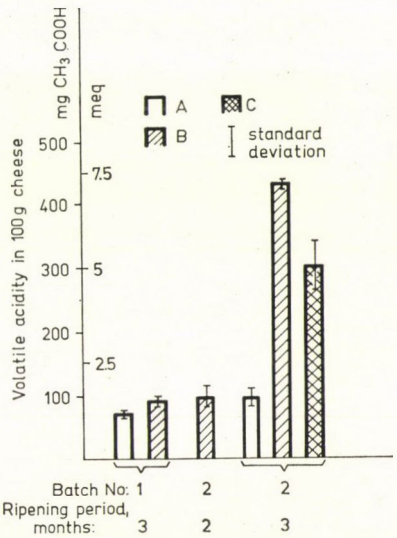

Fig. 4. Free volatile acids (VA) of Emmental cheese prepared with calf rennet and microbial renneting preparations after different periods of ripening. Conditions as in Fig. 2; 420 ml of distillate are collected for VA contents up to and 840 for those above 1.7 meq in 100 g cheese. *A:* cheese manufactured with calf rennet, *B:* cheese manufactured with powdered and *C:* with liquid microbial preparation

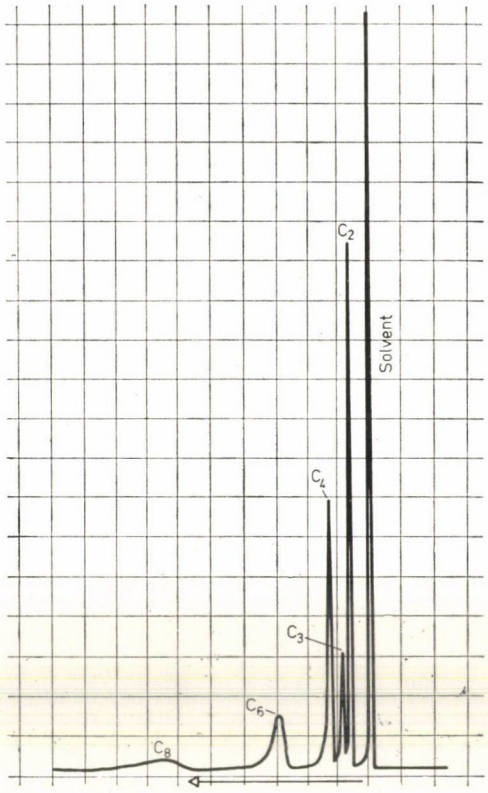

Fig. 5. Chromatogram of volatile acids (VA) after 3 months of ripening of Emmental cheese manufactured with calf rennet (Batch No. 1). Conditions: 1 μl of VA extract obtained by steam distillation as in Fig. 2, with subsequent liberation of acids from Na-salts by cc. H_2SO_4 under diethyl ether, transferring to ether, elimination of water by anhydrous Na_2SO_4 and evaporation of solvent. Chromatography as in Fig. 1. Acids: C_2 = acetic, C_3 = propionic, C_4 = butyric, C_6 = caproic, C_8 = caprylic. →: direction of recorder paper movement

with calf rennet butyric acid, in the one prepared with the microbial enzyme, propionic acid was another major component.

A comparison of the relative amounts of the individual acids in the two cheeses is given in Fig. 7.

In both cheeses acetic acid amounts to somewhat more than 40% of the total volatile acid content, the values do not differ significantly. Similarly, there is no significant difference either between the relative caprylic acid contents which constitute about 10% of free VA. Propionic, butyric and caproic acids were present in the two cheeses in very different percentages: in the one prepared with calf rennet, propionic acid constitutes scarcely more than 10% of free VA content, while in the one obtained with the microbial

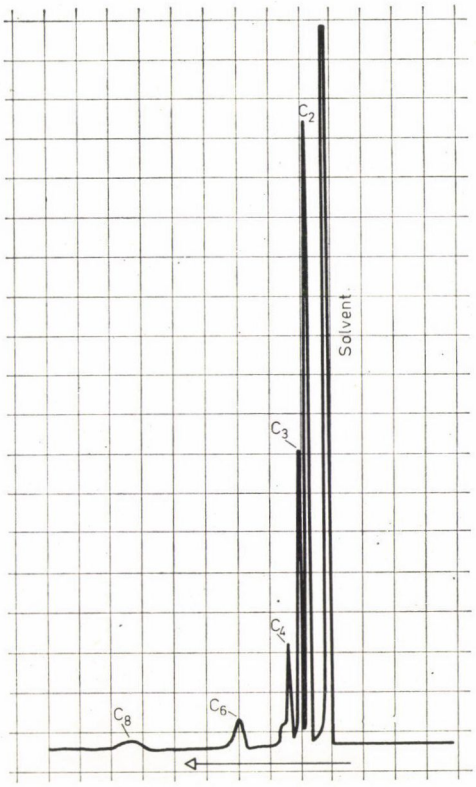

Fig. 6. Chromatogram of VA after 3 months of ripening of Emmental cheese manufactured with powdered microbial rennet (Batch No. 1). Conditions as in Fig. 5 with the exception that 840 ml of distillate have been collected on steam distillation of 50 g cheese. Acids as in Fig. 5. →: direction of recorder paper movement

enzyme it amounts to 30%. The respective values for butyric acid are 21 and 11, and for caproic 14 and 7%.

The volatile acid concentrations of the same samples as expressed in mg in 100 g cheese are represented in Fig. 8.

In cheese manufactured with the microbial preparation propionic acid concentration was more than threefold as compared to that obtained with calf rennet, whereas butyric and caproic acid contents in the latter sample were about 1.5 times as high as in the former one. Acetic and caprylic acid concentrations did not differ significantly in the two samples.

Even more striking differences were found with respect to volatile acid composition in the cheeses from batch No. 2, produced in parallel with calf rennet and with microbial rennet, respectively (Figs. 9 and 10).

As can be seen in Fig. 9, cheese manufactured with calf rennet contained acetic, propionic, butyric and caproic acids, while only acetic and propionic

acids were found in the sample obtained with the microbial preparation. The latter constituted about 90% of free VA. No significant differences were found between the percentages of the corresponding volatile acids of cheese manufactured with the liquid and powdered microbial preparations, respectively. Cheese samples of batch No. 2 manufactured with either milk clotting

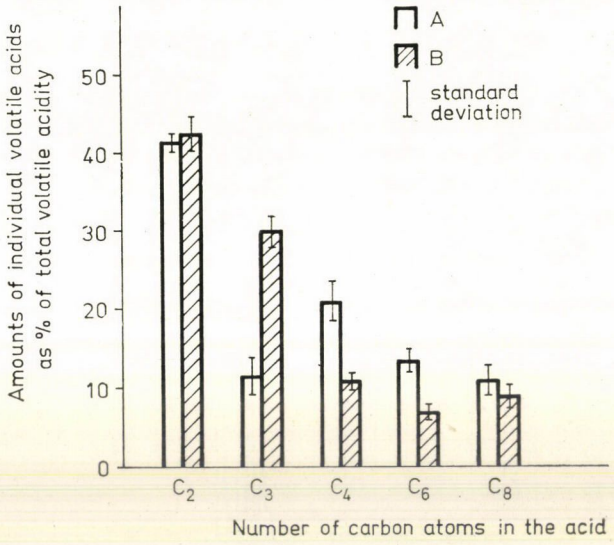

Fig. 7. Relative amounts of VA after 3 months of ripening of Emmental cheese manufactured with calf rennet (*A*) and with powdered microbial rennet (*B*) (Batch No. 1). Conditions as in Figs. 5 and 6, respectively

preparation showed considerable deviations in volatile acid composition from the corresponding samples of batch No. 1: in the samples from batch No. 1 acetic acid amounted to 40% of the total volatile acid content, while in cheese from batch No. 2 it constituted but 25% in the control sample and 10% in the samples obtained with the two kinds of microbial preparations.

Contrary to volatile acid composition, the concentrations of the individual acids as expressed in mg in 100 g cheese and represented in Fig. 10 are rather different in the samples manufactured in parallel with the liquid and the powdered microbial preparation, due to the differences in total free VA content.

Finally, it is interesting to compare the volatile acid composition of cheese manufactured with the powdered microbial preparation at two stages of ripening, *viz.* after 2 and 3 months. In Fig. 11 it can be seen that at both stages free VA content was composed exclusively of acetic and propionic acid, but the ratio of these compounds changed considerably during ripening. After two months acetic acid constituted nearly 80, while after three months

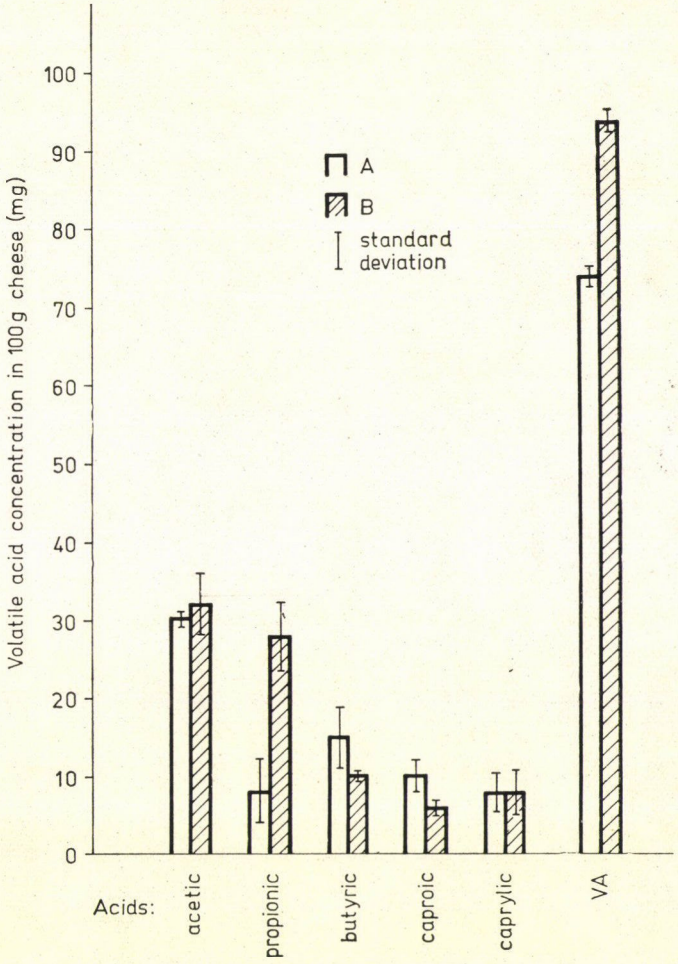

Fig. 8. Concentrations of individual VA and total free VA content after 3 months of ripening of Emmental cheese manufactured with calf rennet (*A*) and with powdered microbial rennet (*B*) (Batch No. 1). Conditions as in Figs. 5 and 6, respectively

only 10% of total free volatile acidity. Free VA content increased roughly 4 times during this period (Fig. 2), thus propionic acid concentration in cheese reached a 16-fold value, while acetic acid content decreased by nearly 50%.

3. Conclusions

3.1. The chromatographic method

The method applied to determine free volatile acidity as well as to prepare the volatile acid extract was developed partly on the basis of literature data

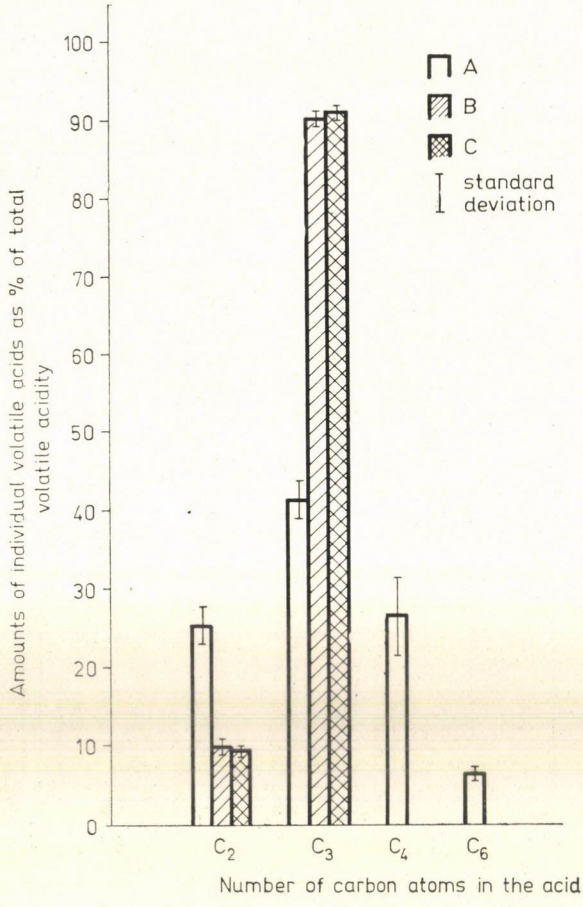

Fig. 9. Relative amounts of VA after 3 months of ripening of Emmental cheese manufactured with calf rennet (*A*) and with powdered (*B*) and liquid (*C*) microbial rennet (Batch No. 2). Conditions of analysis as in Figs. 5 and 6, respectively

(JAGER, 1967; EDWARDS *et al.*, 1971) and partly on the basis of experiments carried out in this laboratory (EL-NOCKRASHY & VÁMOS-VIGYÁZÓ, 1973). Analysis of variance of the data obtained with the first cheese batch confirmed the satisfactory reproducibility of the method. (No significant differences were found between the amounts of individual acids as calculated from the three chromatograms obtained in parallel from the same VA extract and the chromatograms obtained with separate cheese sampling and steam distillation — with the exception of the values found for propionic acid in the cheese manufactured with calf rennet.)

The most time consuming step of the analysis is steam distillation. It was, therefore, considered to replace it by water extraction of VA (LEDFORD,

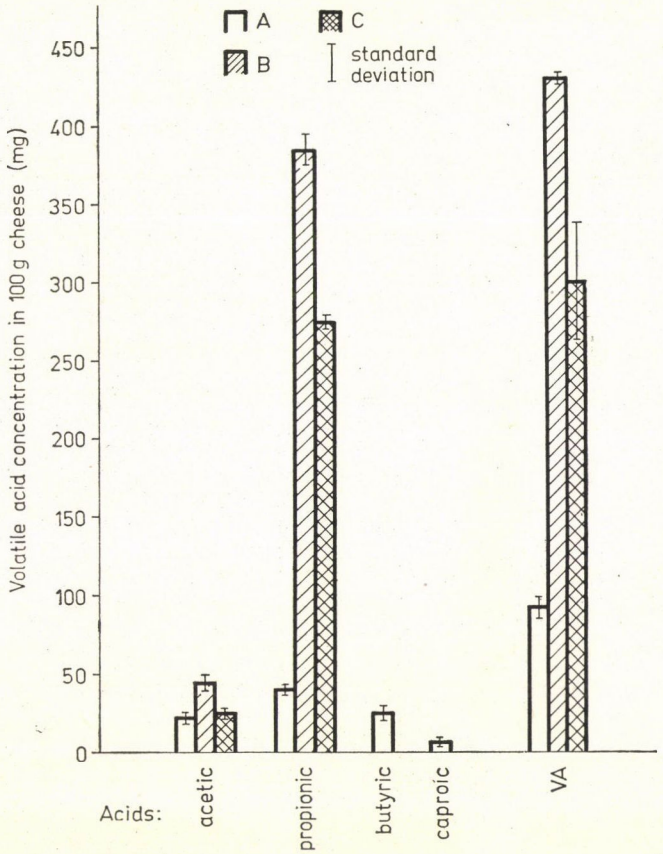

Fig. 10. Concentrations of individual VA and total free VA content after 3 months of ripening of Emmental cheese manufactured with calf rennet (*A*) and with powdered (*B*) and liquid (*C*) microbial rennet (Batch No. 2). Conditions of analysis as in Figs. 5 and 6, respectively

1969; EDWARDS *et al.*, 1971; HAMILTON & RAIE, 1971). With this procedure, however, caproic and caprylic acids could not be extracted in detectable amounts.

Considerable saving of time could be achieved without significant alteration of the distribution pattern of VA by disregarding their last 15% on steam distillation. These findings are in agreement with the data obtained by VANDENHEUVEL (1964) on steam distilling model mixtures of acetic, propionic and butyric acid solutions in the presence of NaCl. According to this author, while 4 volumes of distillate contained all the butyric acid present in the original solution, 10 volumes were necessary to eliminate acetic acid. This phenomenon which was attributed to hydration could be compensated by

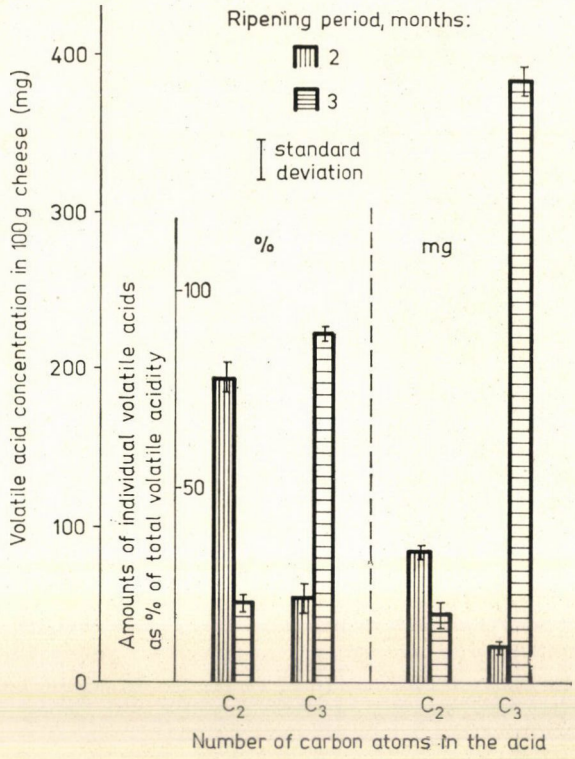

Fig. 11. Variations in VA composition during ripening of Emmental cheese manufactured with powdered microbial rennet (Batch No. 2). Conditions of analysis as in Fig. 6

adding NaCl to the volatile acid solution. Salt content of cheese may have had a similar effect in the present case.

It is an essential feature of the method that free volatile acidity, on which quantitative estimation of the individual acids is based, should be determined from the same steam distillate which is used for chromatographic analysis. Thus errors due to inhomogeneity of cheese may be reduced.

Another source of error in quantitative estimation of VA content is due to the fact that the CO_2 content of cheese has not been eliminated by preliminary operations and thus causes an uncontrollable increase in titrated free volatile acidity. In order to avoid this error estimation of VA concentrations may be carried out also on the basis of calibration curves obtained by chromatographing concentration series of the individual acids (LEDFORD, 1969). This method is, however, subject to considerable error due to residual solvent in the sample to be injected. Taking into account the uncertainty in estimating the concentrations, conclusions will be based primarily on the relative amounts of the acids separated by gas-liquid chromatography.

3.2. Volatile acids of Emmental cheese

The results presented allow to conclude that under identical manufacturing conditions (including raw material, starter culture, production technology and ripening) the clotting enzyme exhibits a marked influence on the VA composition of the product. In cheese produced with microbial rennet propionic acid content is considerably higher as compared to cheese obtained with calf rennet, while butyric acid is formed to a lesser degree or not at all. In the batches investigated cheeses renneted with the microbial preparations had also significantly higher total free volatile acid contents than the respective samples manufactured with calf rennet. These results have, however, to be confirmed by further experiments, especially as considerable differences were found between the two cheese batches produced with calf rennet, too.

The identical volatile acid patterns of the cheese samples obtained with the liquid and powdered forms of microbial rennet, point to the equivalence of the two preparations.

The considerable differences found in the volatile acid composition, especially in the ratio of acetic and propionic acids of the two cheese batches manufactured with calf rennet may, perhaps, be ascribed to the fact that — for reasons not connected with this work — with batch No. 1 a retarded and with batch No. 2 an accelerated ripening procedure has been applied at the plant.

VA composition of cheese obviously depends to a great extent on the starter, the ripening period as well as on the protein and fat content of milk. This may account for the differences and contradictions between the data reported on the subject by various authors. BALOGH (1966) detected acetic, butyric, caproic and caprylic acids in blue cheese, while valeric acid was found in addition to these in Tilsit cheese (BUDSLAWSKI & ROZOWICZ, 1965). No propionic acid was found in Port Salut (Trappist) cheese (WILLART, 1956) in agreement with the authors' own findings (VÁMOS-VIGYÁZÓ & KISS-KUTZ, 1972). Literature data concerning Emmental cheese are divergent, too: some authors reported the presence of acetic, propionic, butyric and isobutyric (PANOUSE *et al.*, 1972), others that of formic, acetic, propionic and butyric acids (JAGER, 1967). A recent qualitative study (NEY & WIROTAMA, 1972) reports on a total of 28 monocarboxylic acids as found in Emmental cheese, among which isocaproic and heptanoic acids have not been mentioned by others. In some cases valeric acid was found as well (MAYR, 1969), while others stressed the absence of this compound. The presence of 2-methyl butyric and, in part of the samples analysed, 3-methyl butyric acid was revealed by LANGLER and DAY (1966). Some authors found no caproic and only traces of n-butyric acid beside measurable amounts of caprylic acid (SCHORMÜLLER & LANGNER, 1960).

Formic acid cannot be detected by the method applied in this study owing to the use of a flame ionisation detector in GLC, but — since this acid is reported to occur mainly in aged cheese (after 5 1/2 months of ripening and storage, respectively; MAYR, 1969) — its presence in the samples investigated is not probable.

Available data as to volatile acid content of Emmental cheese are sparse. Those reported by MAYR (1969) from different sources vary between 330 and 998 mg in 100 g cheese. The corresponding values for the main component, propionic acid, vary from 230 to 470 mg in 100 g cheese (KIERMEIER *et al.*, 1968 a, b; MAYR, 1969). The ratio of propionic to acetic acid was found to be 83 : 7 and 77 : 6, respectively, in samples from different countries (PATTON, 1964), while striking differences were observed in this respect in samples from the same country (LANGLER & DAY, 1966): in most of the samples propionic acid was present in prevailing amounts, however, one of the five cheeses investigated was reported to have a higher acetic than propionic acid content, similarly to batch No. 1 in the present work. The values established in this study for cheese manufactured with calf rennet are considerably lower with respect to both VA and propionic acid content than those reported by MAYR (1969). It must be borne in mind, however, that only free acids have been extracted by the method applied. It seems indicated to extend further investigations also to those present as salts.

No data have been found so far as to the effect of the renneting agent on volatile acid composition. Since the amounts as well as the distribution of VA are subject to wide variations even when using exlusively calf rennet, the effect of the clotting enzyme can be evaluated only by comparison of cheeses manufactured from the same raw material and — with the exception of the clotting preparation — under strictly identical conditions, as was done in the present study.

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TURBIDIMETRIC ACTIVITY ASSAY METHOD FOR THE DETERMINATION OF THE EFFICIENCY OF CELLULASE PREPARATIONS

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The cellulose-hydrolysing performance of cellulolytic enzyme preparations was measured on the basis of changes in turbidity in a suspension of a water-insoluble crystalline cellulose. Linear correlation was observed to exist between the reduction of turbidity and the reaction time in the interval between 0 and 60 minutes in case a substrate concentration of 0.05% (w/v) and enzyme concentrations between 0.05 and 0.15% were applied. After a certain period of induction, linear correlation exists between the turbidimetric data measured and the quantity of glucose formed during hydrolysis, thus, the activity of the enzyme may be expressed as μmole glucose formed in the reaction mixture per minute.

Measuring the cellulolytic activity of enzyme preparations by chemical methods involves difficulties. Generally the methods of measurement are based on the determination of the reducing substances formed in the course of hydrolysis, irrespective of whether the substrate is a native or a modified cellulose derivative. Native celluloses are highly resistant to enzymic hydrolysis, thus chemical changes are hardly measurable. On the other hand activity measurement on solubilized cellulose derivatives is not unambiguously characteristic of the preparation, from the practical point of view.

Therefore, to measure the activity of cellulase preparations a method has to be found which is based on a physical change simply measurable with high accuracy and convertible to chemical dimensions (VAS, 1971).

The turbidimetric method developed on the basis of the investigations of FLORA (1965) complies with these requirements. The performance of the "hydrocellulase" component of the cellulase enzyme complex is measured on the crystalline hydro-cellulose, the residue of pure cotton wool hydrolysed in a mild acidic solution. As an effect of the cellulase preparation the turbidity of the hydro-cellulose solution is reduced.

The aim of this study was to express numerically, in the internationally accepted chemical units (μmoles per minute) the activity of cellulase preparations, by establishing the correlation between the turbidimetric data and the amount per minute of converted hydro-cellulose, *i.e.* the amount of glucose formed during hydrolysis.

1. Materials and methods

1.1. Materials

1.1.1. Substrate. 25 g of cleaned cotton waste (sterile cotton, used in ophthalmology) was soaked in 500 ml 10.8 *N* hydrochloric acid for 48 hours and from time to time thoroughly shaken. The suspension, formed during 48 hours, was then whashed with distilled water in excess, decanted and neutralized with $NaHCO_3$ and washed with distilled water till free from chloride (FLORA, 1965).

The neutralized and chloride-free suspension was dried at 105 °C to constant weight. The cellulose concentration thus measured was 0.08 $g \cdot ml^{-1}$.

The hydro-cellulose suspension thus prepared is a stable product and may be kept for several years.

1.1.2. Enzyme preparation. Meicelase P preparation, obtained by the Meiji Seika Kaisha firm, Japan, from *Trichoderma viride* cultures was used as model enzyme.

The filter paper disintegration performance of the preparation was found to be 292 $\pm$ 7 units. This value is understood to mean that from the 1% Macherey & Nagel MN 60 filter paper substrate of pH 4.8 one g enzyme preparation liberates 0.292 g reducing substance during 60 minutes at 40 °C (NIWA & NAKAMURA, 1965).

1.1.3. Buffer. The enzyme solutions and the reaction mixtures were prepared with a 0.1 *M* citrate-phosphate buffer of pH 4.8 (DAWSON *et al.*, 1959).

1.2. Methods

The underlying principle of the method is that the reduction of turbidity in a finely dispersed hydro-cellulose suspension, as induced by the cellulase preparation, is followed up by the measurement of changes in the light absorbance values.

Every sample contained 5 ml substrate, 4 ml buffer solution, and 1 ml enzyme solution.

The reaction mixture was weighed into 100-ml Erlenmeyer flasks, these were stoppered, then shaken at 40 °C and 250 rpm. The absorbance values *(A)* were measured against distilled water after reaction periods of 0, 15, 30, 45, 60, 90 and 120 minutes, resp.

The reduction of turbidity (ΔA) was calculated from the values as measured at 0 and the given reaction time.

In order to be able to select the appropriate wavelength the absorption spectra of reaction mixtures of varied hydro-cellulose and enzyme content were established with a Perkin—Elmer 137 UV type automatic spectrophotom-

eter in the range of 350—750 nm. Since in the range studied the white opalescent samples, or those gaining pale yellow discoloration from the enzyme solution, had no absorption maxima, measurements could be carried out at any wavelength in the visible spectrum.

Changes in turbidity were measured with a Spektromom spectrophotometer, type 203. The differences in the values as measured at wavelengths 400, 500, 700 and 800 nm, respectively, were not significant, thus subsequent measurements were carried out at 700 nm, arbitrarily chosen.

The substrate concentration most appropriate from the point of view of measuring technique was determined from the hydro-cellulose calibration curve.

As seen in Fig. 1, solutions of 0.015—0.075% (w/v) hydro-cellulose concentration were most suitable for absorbance measurement. Since in the course of the reaction the absorbance values decrease, it is expedient to use, initially, hydro-cellulose solutions of higher concentration. However, the use of substrates of very high concentration may cause sedimentation of hydro-cellulose during measurement.

To select the substrate and enzyme concentration most suitable for studies on enzyme kinetics, investigations were carried out at the following concentrations:

Hydro-cellulose concentration, S (%, w/v)	Meicelase P concentration, E (%, w/v)
0.015	0.050
0.025	0.075
0.040	0.100
0.050	0.150
0.065	0.250
0.080	0.500
0.100	

The hydro-cellulose solutions were prepared with distilled water, the enzyme solutions with buffer solution (para. 1.1.3.).

After combination of the components, and after different incubation periods the hydrogen ion concentration of the reaction mixtures was measured with a Metrohm E 166 type pH-meter. In the time interval of 0 to 120 minutes a pH value of 4.9 was measured.

The glucose content of the reaction mixtures was determined by means of the Glucostat preparation of *Worthington Biochemical Corporation* according to the instructions as described in the manual (1967) of the firm.

Mathematical evaluation of the results was carried out by means of standard deviation calculations and regression analysis (KÖRMENDY, 1964).

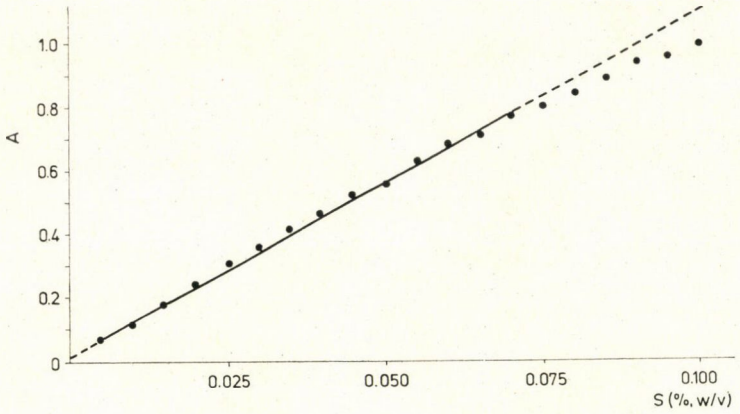

Fig. 1. Absorbance of hydro-cellulose suspensions. Absorbance measured at 700 nm (A) as a function of hydro-cellulose concentration (S). Standard deviation of the points of measurement, based on 6 parallel measurements: between 0.004 and 0.009 A. The coefficient of variation varied between 0.96 and 2.4%. Equation of the regression curve: $A = 0.019 + 0.0549\ S$, $r = 0.999$, $n = 84$. 14 substrate concentrations were taken into consideration in calculating the regression curve

2. Results

For a hydro-cellulose concentration of 0.05% (w/v) and three Meicelase P concentrations (0.05, 0.1, and 0.5%, w/v) reduction in turbidity is shown as a function of reaction time in Fig. 2.

As can be seen in the figure the correlation between reaction time and reduction of turbidity as induced by the enzyme is linear in the interval between 0 and 60 minutes.

The criterion of saturation for the substrate was studied at hydro-cellulose concentrations of 0.015, 0.025, 0.04, 0.05, 0.065, 0.08 and 0.10% (w/v), respectively. The correlation between concentration and reduction of turbidity is shown in Fig. 3.

The investigations prove that the initial hydro-cellulose concentration of 0.05% (w/v) which is satisfactory from the measurement aspect, is also an adequate substrate concentration from the point of view of enzyme kinetics, since by further increasing the value of S the change in the value of turbidity reduction is not significant.

Reduction in turbidity as a function of enzyme concentration is illustrated in Fig. 4.

A more or less linear correlation between decrease in turbidity and enzyme concentration exists in the concentration range of 0 to 0.1% (w/v) enzyme preparation.

Fig. 5 shows the slope of the regression curves, taken as reaction velocity (v) and expressed in $\Delta A \cdot \mathrm{min}^{-1}$, as a function of enzyme concentration.

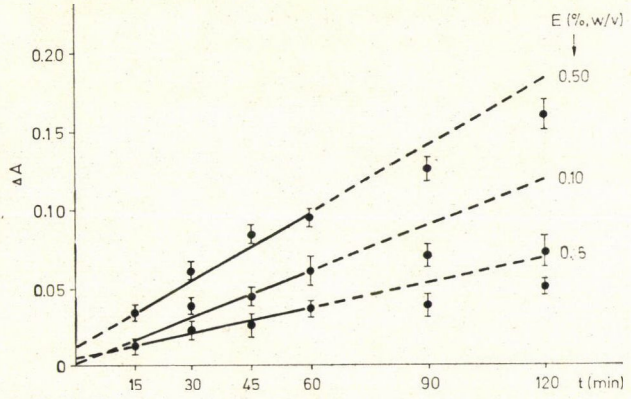

Fig. 2. Cellulase activity of Meicelase P as a function of the reaction time (t). pH = 4.9, T = 40°C. Equations of the regression curves at hydro-cellulose concentration 0.05% (w/v): at enzyme concentration (E): 0.05% (w/v), $\Delta A = 0.0053 + 0.0005$ t, r = 0.952, n = 38; at enzyme concentration 0.1% (w/v): $\Delta A = 0.0019 + 0.00097$ t, r = 0.888, n = 36; at enzyme concentration 0.5% (w/v): $\Delta A = 0.015 + 0.0013$ t, r = 0.968, n = 37. The regression curves were calculated each from 4 points of time and 9—10 individual ΔA values. The vertical bars represent two standard deviations, based on 9—10 parallel results. The standard deviation varied between 0.002 and 0.01 ΔA and the coefficient of variation between 5.3 and 11.6%

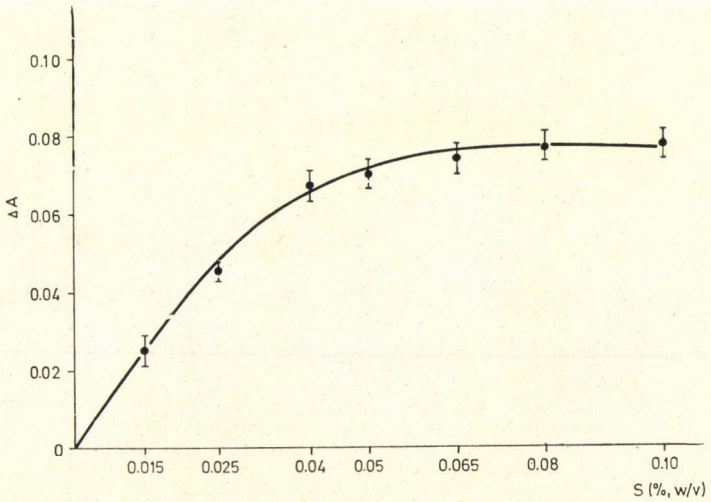

Fig. 3. Correlation between the change in turbidity (ΔA) and the substrate concentration (S) at 60-minute reaction period and a Meicelase P concentration of 0.15% (w/v). The vertical bars represent two standard deviations, based on 9 parallel results. The standard deviation varied between 0.005 and 0.016 ΔA and the coefficient of variation between 7.4 and 9.7%

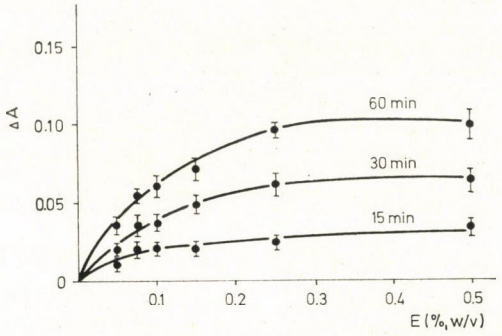

Fig. 4. Activity of the Meicelase P preparation as a function of the enzyme preparation concentration (E) at reaction periods of 15, 30 and 60 minutes, resp. pH = 4.9, T = 40°C. Substrate concentration: $S = 0.05\%$ (w/v) hydro-cellulose. The vertical bars represent two standard deviations, based on 9 parallel results. The standard deviation varied between 0.003 and 0.009 ΔA and the coefficient of variation between 7.6 and 12%

In this case, too, linear correlation is observed in the enzyme concentration range of 0 to 0.1% (w/v).

By plotting the amount of reaction product (represented by ΔA) as a function of the product of enzyme concentration and reaction time (SELWYN, 1965), Fig.6 was obtained. This shows that, at different enzyme concentrations, the experimental points may be fitted to different curves, indicating that some denaturation may occur during the test or some problems of diffusion, multi-enzyme action, *etc.* may prevail.

The correlation between the turbidity value characteristic of the enzymic degradation of hydro-cellulose and the amount of glucose formed in the course of degradation is illustrated in Fig. 7.

As seen in the figure in the absorbance range between 0 and 0.037 no

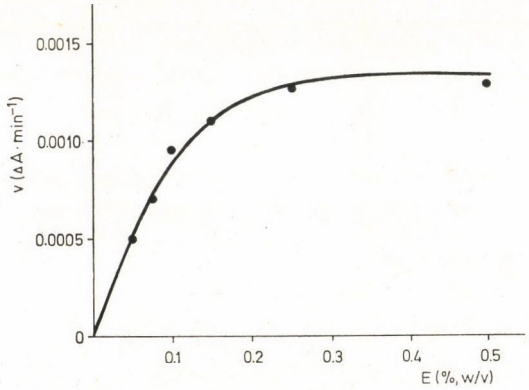

Fig. 5. Correlation between reaction rate (v) and the concentration of the enzyme preparation (E). pH = 4.9, T = 40°C. Substrate concentration: S = 0.05% (w/v) hydro-cellulose

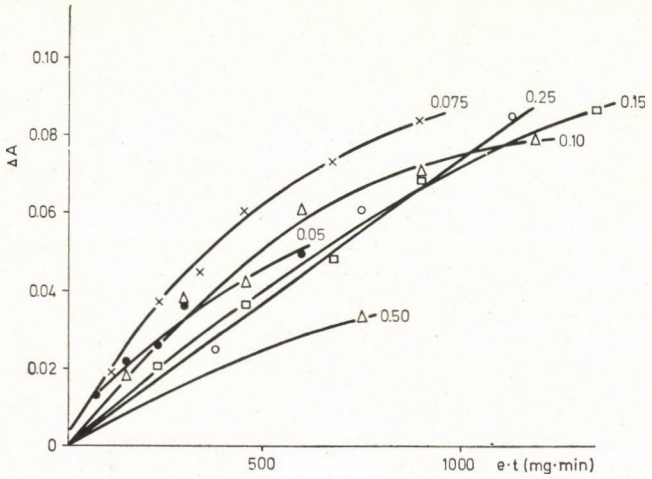

Fig. 6. Correlation between the change in turbidity (ΔA) on one hand, and the product of the amount of enzyme preparation weighed into the reaction mixture (e, mg) and the reaction period (t, min), on the other (SELWYN, 1965). pH = 4.9, T = 40°C. Enzyme concentration levels (E, %, w/v) are indicated by the figures along the curves. Substrate concentration: $S = 0.05\%$ (w/v) hydro-cellulose

glucose is formed as an effect of enzyme action. In this range, probably, hydrocellulose breaks down to units in the oligosaccharide order of magnitude. Above the 0.037 ΔA value the correlation between a reduction in the absorbance value (turbidity) and the glucose formed is linear. On the basis of this correlation it is possible to convert the measured physical changes to chemical dimensions and to express enzyme activity as glucose μmole · min^{-1}.

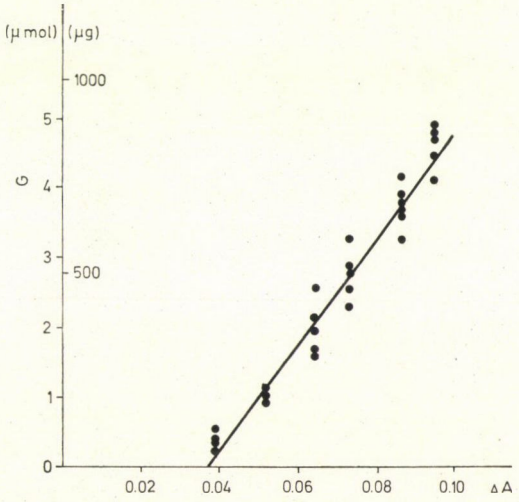

Fig. 7. Correlation between the change in turbidity (ΔA) and the amount of glucose (G) formed during degradation in 10 ml reaction mixture. $S = 0.05\%$ (w/v), $E = 0.10\%$ (w/v). Equation of the regression curve: $G = 13.6 \cdot \Delta A - 0.492$, r = 0.979, n = 30

3. Conclusions

The activity of cellulase preparations in hydro-cellulose substrate is measurable spectrophotometrically (by the measurement of turbidity), because the correlations between reaction time and *(a)* the initial decrease in turbidity and *(b)* the quantity of glucose formed during degradation, are linear.

A convenient substrate concentration of the method is 0.05% (w/v) hydro-cellulose at enzyme concentrations between 0.05 and 0.10% (w/v).

Under these conditions of concentration the criterion for substrate saturation exists (Fig. 3).

Several ways of expressing the enzyme activity exist. One of the ways is that the reduction of hydro-cellulose concentration belonging to a given change in absorbance is calculated from the hydro-cellulose calibration curve (Fig. 1). In the knowledge of this it is easy to calculate the amount of hydro-cellulose converted in the course of one minute.

According to Fig. 2, for instance, at an enzyme concentration of 0.10% (w/v) a change in absorbance of 0.06 corresponds to a 60-minute reaction period. On the basis of the slope of the calibration curve as seen in Fig. 1, a change of 0.00529% (w/v) in the hydro-cellulose concentration corresponds to the above change in absorbance and this means the breakdown of 529 μg hydro-cellulose in 10 ml substrate, *i.e.* 0.01 g of enzyme preparation as present in 10 ml of the reaction mixture has decomposed 8.66 μg hydro-cellulose per minute. If, the molecular weight of the enzyme preparation being unknown, the amount of enzyme causing the breakdown of 1 μg hydro-cellulose per minute is chosen arbitrarily as unit of enzyme activity (1 U) the hydro-cellulose-degrading enzyme concentration of the Meicelase P preparation is 866 $\mu g \cdot min^{-1} \cdot g^{-1}$ ($U \cdot g^{-1}$).

Another way of expressing enzyme activity would be the registration of the amount of glucose formed per minute, using the international unit dimension (Anon, 1965). In case of the example given above in a 10 ml sample of 0.10% (w/v) enzyme concentration (Fig. 2) a 0.06 change of absorbance occurs during the 60-minute reaction period. In accordance with Fig. 7 this signifies the liberation of 315 μg (1.75 μmoles) glucose. Since, as seen in Fig. 7, up to $\Delta A = 0.037$ the formation of glucose is not observed, it seems expedient to count the process of glucose formation in the given case from the corresponding point of time (in Fig. 2 from the 36th min). Thus 1.75 μmoles glucose were liberated during (60−36 =) 24 minutes, and 0.01 g enzyme preparation present in the 10 ml reaction mixture catalyzed the formation of (1.75 : 24 =) 0.072 μmole glucose per minute. The glucose-forming enzyme concentration of the preparation is, therefore, 7.2 μmoles $\cdot min^{-1} \cdot g^{-1}$ ($IU \cdot g^{-1}$). If the induction period of glucose formation (in this case 36 minutes) would not be account-

ed for, the enzyme concentration would amount to $\left(\frac{1.75}{60 \cdot 0.01} =\right)$ 2.92 μmoles$\cdot$min$^{-1}\cdot$g^{-1} (IU$\cdot$g^{-1}).

It is evident that the commercial enzyme preparation used in the experiments contains not only one enzyme and a clear picture of the hydrolysis of hydro-cellulose, or of the formation of glucose could be obtained only using pure preparations. In this work the method described here may render assistance.

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EFFECT OF HEAT TREATMENT ON THE NUTRITIVE VALUE OF PROTEINS IN MILK POWDER

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In the course of model experiments skim milk powder of 2.45 and 5.70% moisture content were heated in a temperature range between 60°C and 120°C. For the reactions occurring during heating formal kinetic expressions were derived, based on the experiments. The decomposition of lysine in the milk powder protein was characterized by a reaction of fourth order as a partial reaction while that of tryptophan by a reaction of first order.

Similarly to the formation of 5-hydroxy-methylfurfural, the decomposition of lysine depends to a greater extent on the moisture content of milk powder than does the decomposition of tryptophan.

The different behaviour of lysine and tryptophan during heating permits of the conclusion that lysine takes part in a Maillard-type reaction while tryptophan undergoes either thermolysis or other, perhaps oxydative decomposition. In spite of this, the presence of carbohydrates contributes to the decomposition of tryptophan.

In the milk powder of lower moisture content the activation energy of the examined processes (formation of 5-hydroxy-methylfurfural, decomposition of lysine and tryptophan) was greater than that in the milk powder of higher moisture content.

The change of the relative nutritive value of proteins as determined with the protozoa strain *Tetrahymena pyriformis* W advances approximately in parallel with the decomposition of the limiting amino acids. In the case of a milder heat treatment the limiting methionine is rather stationary, at a higher temperature tryptophan takes over the limiting role and the nutritive value of protein decreases considerably.

Milk powder is the most frequently applied form of preserved milk. It has been an important basic material of infant formulas and baby foods for a long time. Further, it plays an important role in animal feeding and as an additive in the food industry. During storage of the milk powder a reaction may take place between the protein and the sugars, known for long as the Maillard-reaction, which reduces the nutritive value of protein and causes browning and other organoleptic changes in the milk powder. Of the great number of research papers on the subject, published in Hungary, the work of TELEGDY KOVÁTS (1954) and of SPANYÁR (1957) shall be mentioned here.

The moisture content of milk powder exerts a considerable influence on the factors determining the nutritive value of protein during storage. This concerns first of all lysine. During a 10-week storage period at a temperature of 40 °C, ERBERSDOBLER (1970) found a small decrease of the available

lysine, at a moisture content of 4%. When increasing the moisture content, the level of available lysine decreased considerably. According to his observations a moisture content of 8 per cent causes the maximal damaging effect, above this level the quantity of available lysine increases again. In the course of previous storage experiments (DWORSCHÁK, 1971) it was pointed out that by raising the moisture content in milk powder the 5-hydroxy-methylfurfural content, showing in many cases a parallelism with the Maillard-reaction, increased and the quantity of available lysine fell. This was observed both in Hungarian and in foreign milk powders. These results are in accordance with the data of LINDNER (1951) who found that the solubility of milk powder with a moisture content above 4 per cent decreased gradually because of marked changes in the protein.

It was decided to carry out some model experiments to gather information on processes taking place in the milk powder.

The conditions of treatment were chosen so as to keep changes originated from production and storage at a level insignificant in comparison to those caused by the experimental conditions. In the experiments extensive heat treatment was applied to throw light on the correlations between heat treatment and the nutritive value of protein in the given milk protein and lactose system. Heat treatments of similar extent are applied, for example, to determine the date of expiration of pharmaceuticals (ZAJTA, 1962), and in many fields of food science. The investigations were extended over dried egg-powder containing small quantities of carbohydrates.

1. Materials and methods

1.1. Materials

1.1.1. Milk powder. Skim milk powder, not older than 3 months, was purchased from the Milk Processing Plant, Berettyóújfalu. The moisture content of the milk powder was 5.70%. Half of the sample was used directly for heating, and the other half was dried at room temperature in a vacuum drying oven, till the moisture content decreased to 2.45%.

1.1.2. Dried eggs. A product of the Hungarian Poultry Industry of a moisture content of 2.8 per cent.

1.1.3. Determination of the moisture content of the samples. The moisture content of the unheated milk powder was determined in accordance with Hungarian Standard MSZ 3726/2—68 by drying at a temperature of 102 °C to constant weight.

1.1.4. Heating of the milk powder and of dried eggs. 10 g each of milk powder of the two different kinds and of dried eggs were placed into glass ampullae,

closed, and heated in an aluminium block thermostat (Type 615 KUTESz, Budapest). Temperatures applied were in the range between 60 and 120 °C. Treatment times varied from one to four hours. Altogether 40 samples were treated.

1.2. Methods

1.2.1. Hydrolysis of proteins. The hydrolysis of the proteins was carried out according to LINDNER and co-workers (1957) with 20% hydrochloric acid at 105 °C. The method of the same authors was followed in the treatment of the hydrolysates.

1.2.2. Chromatographic separation of amino acids and their determination. As to the paper and thin-layer chromatography of amino acids we refer to the works of LINDNER and co-workers (1957) and to an earlier paper of one of us (DWORSCHÁK, 1970). The separation of lysine was carried out by the method of DÉVÉNYI and co-workers (1971) on *Fixion* 50×8 plate. The spots developed by ninhydrin were evaluated with a *Chromoscan* densitometer *(Joyce—Loebl,* England).

1.2.3. Tryptophan. Tryptophan was determined by the Spies-Chambers method with p-dimethylamino-benzaldehyde reagent, photometrically (GREENSTEIN & WINITZ, 1961).

1.2.4. The relative nutritive value of protein. This determination was carried out according to the method of HEGEDÜS (1971) with the protozoa strain *Tetrahymena pyriformis* W. The procedure applied is the improvement of the methods of STOTT and SMITH (1963) and that of BAUM and HAENEL (1965).

1.2.5. 5-Hydroxy-methylfurfural (HMF). The determination of free and total HMF (bound and free) obtained by oxalic acid hydrolysis, was carried out by the thiobarbituric acid method elaborated by KEENEY and BASSETTE (1959) for milk and milk products. Bound HMF is understood to be HMF set free from the 3-deoxy-hexosone and 3,4-unsaturated hexosone.

1.2.6. Unsaturated dicarbonyl compounds. The unsaturated dicarbonyl compounds were determined according to COLE (1967) based on the extinction values at 280 nm *(Spektromom* 203, Budapest). The extinction values are not specific, because other compounds, for example HMF, also absorb light at this wavelength.

1.2.7. Evaluation of the data. Formal kinetic equations were derived from the results as far as it was possible. Determination of the order of reaction, of the rate constants, of the half-life periods and of the activation energies was performed by the differential method described by ERDEY-GRÚZ (1972). The values obtained were valuated by the method of least squares. A confidence level of 90% was applied.

2. Results

2.1. Determination of certain formal kinetic characteristics

Fig. 1 shows the change of lysine in the protein as a function of the time of heat treatment. It can be seen from the diagram that — at a certain temperature — decomposition of lysine in milk powder of higher (5.70%) moisture content takes place much more intensively than in milk powder of 2.45% moisture content.

The degradation of tryptophan as a function of time can be seen in Fig. 2. Here, too, the dependence of the rate of decomposition on the moisture content can be observed qualitatively. However, the form of the curves differs considerably from those observed in the case of lysine. In the latter case the curves approximate a limiting value, while in most cases the tryptophan content approximates zero.

Under the given conditions of heat treatment a decrease of 15—20% was observed with another essential amino acid, methionine.

The results correspond to data found in the literature (ERBERSDOBLER & DÜMMER, 1971) with the exception of those related to heat damage to tryptophan which was either not examined or found non-existent by most of the authors. Degradation of tryptophan was found more extensive than that of lysine when fish-protein (PELROY & SPINELLI, 1971) or soy proteins (BADENHOP & HACKLER, 1971) were heated.

Formal kinetic equations were derived on the basis of curves of lysine and of tryptophan, and the reaction order was established from them. The

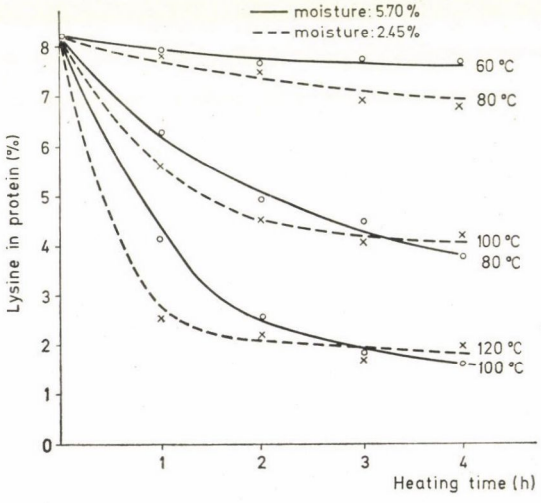

Fig. 1. Decomposition of lysine in the protein of heated milk powder

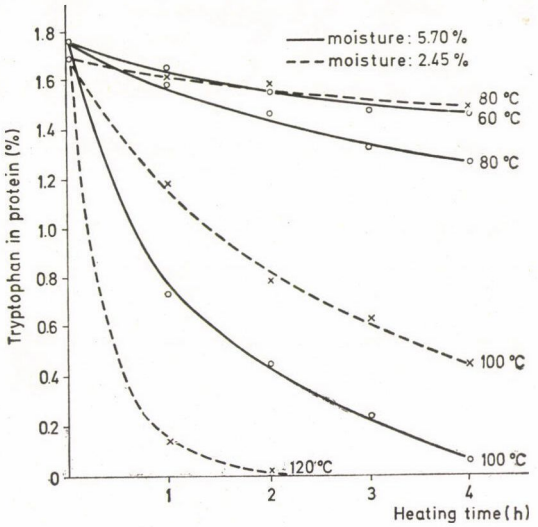

Fig. 2. Decomposition of tryptophan in the potein of heated milk powder

overall order of the reaction could not be established since the reaction between lactose and some amino acids was not known. As seen in Fig. 3 the decomposition of lysine conforms to a reaction of fourth order, while that of tryptophan to a reaction of first order. The equation of HMF formation is shown in the same figure, and according to this both the formation of free and total HMF is directly proportional to time.

The rate constants of the equations were determined and their logarithm was plotted as a function of the reciprocal of temperature. As far as it was possible to derive with sufficient confidence the equation of a straight line of the points obtained it was used to calculate the activation energies of the

LYSINE $\frac{d[Lys]}{dt} = -k \cdot [Lys]^4$
TRYPTOPHAN $\frac{d[Trp]}{dt} = -k \cdot [Trp]$
5-HYDROXY-METHYLFURFURAL (HMF) $\frac{[HMF]}{t} = k \cdot \underbrace{[Lactose]^x \cdot [Protein]^y}_{const.}$

Fig. 3. Formal kinetic expressions for lysine, tryptophan and HMF in heated milk powders

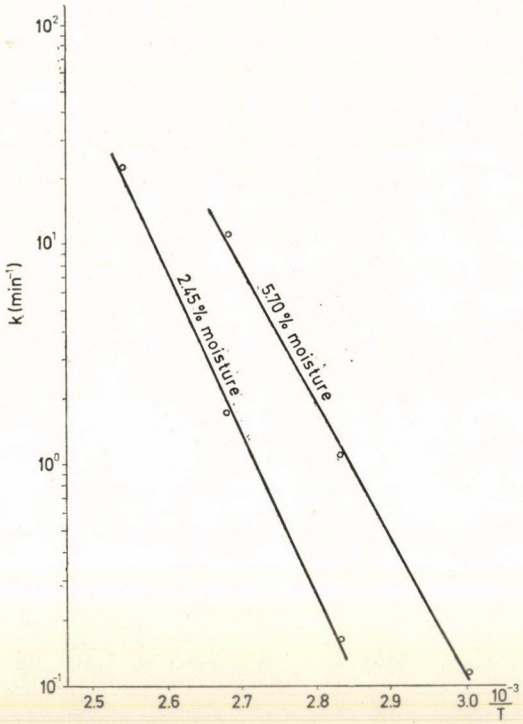

Fig. 4. log k and $1/T$ values of lysine in heated milk powders

reactions in the temperature range examined. An example of this, graphically presented, is the curve of lysine decomposition (Fig. 4).

The activation energies of the reactions can be seen in Table 1. In spite of the relatively low confidence of data it can be seen that the energy values belonging to samples of lower moisture content are higher. The formation of HMF in milk powder of 5.70% moisture content needs nearly the same activation energy as was found for sugars (ÖRSI, 1971; DWORSCHÁK & ERDÉLYI, 1967).

Table 1

Activation energies of reactions in milk powder (Kcal mol^{-1})

	Character of reaction	Moisture cont. 5.70%	Moisture cont. 2.45%
5-hydroxy-methylfurfural (free)	formation	34.8±12.7	43.6±4.5
5-hydroxy-methylfurfural (total)	formation	28.8±13.2	38.4±5.7
Unsaturated carbonyl compounds	formation		30.9±6.9
Lysine	decomposition	28.6± 6.1	33.2±7.5
Tryptophan	decomposition		28.3±4.3

The correlation between the reaction rates and the change of the moisture content was characterized by the quotient of the rate constants of the two different samples. As seen in Table 2, while the moisture content of the milk powder increased from 2.45% to 5.70%, the rate of decomposition of lysine increased six- to seven-fold, and that of tryptophan two and a half-fold.

Table 2

Quotients of rate constants in milk powders of different moisture contents heated to 80°C and 100°C

	$\frac{k_{5.7\%}}{k_{2.45\%}}$	
	80°C	100°C
5-hydroxy-methylfurfural (free)	15.80	7.32
5-hydroxy-methylfurfural (total)	7.80	2.88
Lysine	6.95	6.30
Tryptophan	2.47	2.10

The best way of expressing the effect of heat treatment on lysine and tryptophan is the use of the half-life period of the decomposition reactions (Table 3). When heating milk powder of 5.70% moisture content at 80 °C and 100 °C, lysine decomposes at a higher rate than tryptophan, while in a

Table 3

Half-life period of lysine and tryptophan decomposition (minutes)

	60°C	80°C	100°C	120°C	140°C
Lysine					
in milk powder					
of 5.70% moisture content	2 820±624	289±83	29±7.8		
in milk powder					
of 2.45% moisture content		2 010±478	182±36	14±2.3	
in dried eggs					
of 2.8% moisture content					356±80
Tryptophan					
in milk powder					
of 5.70% moisture content	691±115	450±62	57±9.8		
in milk powder					
of 2.45% moisture content		1 110±230	118±17	18±3.2	
in dried eggs					
of 2.8% moisture content				227±35	

milk powder of 2.45% moisture content the situation is inverse. The reason for this is the different dependence of the rate constants on moisture content.

The half-life period values belonging to the decomposition reactions of lysine and tryptophan upon heat treatment of dried egg-powder are also shown in Table 3. In dried eggs, which is practically free of sugar, the decomposition of both amino acids takes place according to a reaction of first order. The half-life period of lysine is even at the temperature of 140 °C considerably higher than that of tryptophan heated to 120 °C.

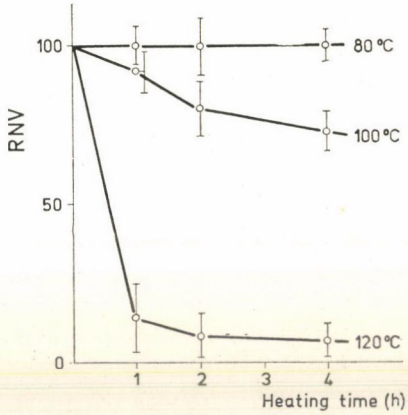

Fig. 5. Changes in the relative nutritive value (RNV) of the proteins of milk powder upon heat treatment. The bars indicate standard deviations

2.2. Investigation of the relative nutritive value (RNV) of proteins by a microbiological method

The investigations were carried out with a milk powder of 2.45% moisture content. The results can be seen in Fig. 5. At a temperature of 80 °C there was no change in the protein value, while at 100 °C the change was considerable, and even more extensive at 120 °C. In this latter case the diagram reminds of the decomposition curve of tryptophan and lysine.

3. Conclusions

Based on the formal kinetic expressions and other characteristics as regards the decomposition of lysine and tryptophan, and the formation of HMF the following conclusions can be drawn. In Fig. 3 the lactose and protein concentrations, determinative of the formation of HMF may be considered constant since HMF is not formed directly from these, but it is the end-product of consecutive reactions in several steps (ANET, 1965). The decrease in the concentration of the original materials had no influence on the formation of the end-product.

The participation of lysine in the decomposition reaction conforms to the fourth power, and this infers the active role of a foreign molecule, in the present case that of lactose. As regards tryptophan, the reaction being of first order refers to the participation of fewer external reactants. In this connection, the results summarized in Table 3 show the difference between the heat treatment of milk-powder and dried eggs, and verify that the presence of sugar contributes to the decomposition of lysine to a greater extent than to that of tryptophan. In dried eggs — due to lack of carbohydrates — also the order of the lysine reaction is decreased.

The correlation between the moisture content and the quotient of rate constants (Table 2) emphasizes the difference in the mechanism of lysine and tryptophan decomposition once more. The quotients obtained in the case of lysine are nearer to the HMF values, characteristic of the Maillard-reaction, than those of tryptophan.

All the results prove consistently that lysine reacts with lactose according to the Maillard-reaction. This type of reaction is suggested by the circumstance that the 6-amino group of lysine is not bound in the protein, further on — according to TÄUFEL and co-workers (1956) — it is much more reactive than the amino group in the 2nd position.

The imine group being on the indole ring of tryptophan has no nucleophilic character, and therefore it is not likely to participate in the Maillard-reaction. On the basis of the heat treatment experiments the thermolysis of the indole ring, and perhaps the proceeding of other processes, for example oxidation, is presumed. According to SUNDBERG (1970) peroxides may be formed from indole derivatives in the presence of oxygen, and these decompose by splitting of the indole ring. Under slightly oxidative conditions the indole ring does not split (SUNDBERG, 1970) but oxygen is substituted in the 2nd position, (Fig. 6). Oxygen substitution makes tryptophan unsuitable for reacting with p-dimethylamino-benzaldehyde (FRIEDMAN & FINLEY, 1971) in the course of the *Spies-Chambers* determination.

From the fact that the activation energies in milk powder of a lower moisture content are increased it may be concluded that in these samples the rate of the discussed reactions is lower at a lower temperature than in a milk powder of 5.7% moisture content.

The results obtained for the relative nutritive value of proteins may be traced back to the formation of limiting amino acids in the heated milk

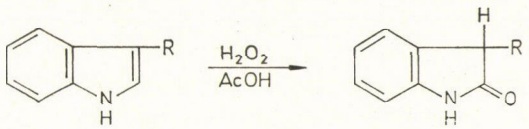

Fig. 6. Oxidation of indole derivatives

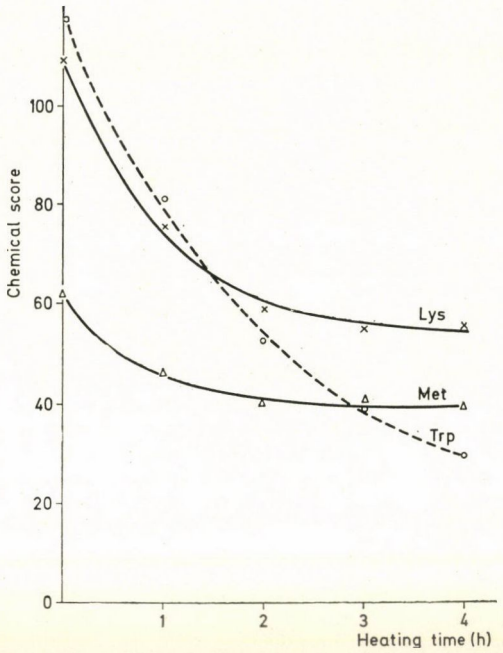

Fig. 7. Changes in limiting amino acids in milk powder of a moisture content of 2.45%, during heating at 100 °C

powder. In the case of a lower heat treatment, at a temperature of 80 °C the limiting amino acid is methionine, and since it is only slightly decomposed, the nutritive value does not change. However, in the case of a heat treatment at 120 °C it is the easily decomposing tryptophan that becomes limiting. Tryptophan takes the limiting role from methionine after a heat treatment of 3 hours at a temperature of 100 °C. This is shown in Fig. 7, where the amino acid contents of the whole egg protein, that is the chemical score values (MITCHELL & BLOCK, 1946) are compared. Changes in the relative nutritive value, caused by heat treatment and measured with *Tetrahymena pyriformis*, are in accordance with the classical principle of limiting amino acids.

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CONTINUOUS HIGH FREQUENCY DIELECTRIC EQUIPMENT AND TECHNOLOGY FOR BAKING BREAD

T. RUNTÁG

(Received March 19, 1973)

In an earlier issue of this periodical we have already reported on our experiments and their results concerning the application of high frequency dielectric fields to the baking of bread (RUNTÁG & DEMECZKY, 1973).

These laboratory-scale experiments gave a promising answer to the posed questions: the bread baked of leavened dough in the high frequency field had a looser structure, with high specific volume and elasticity, and ageing properties more favourable than those of products of the traditional technology. No difference in the taste of the products could be detected.

The laboratory experiments gave an unequivocal answer to the question of applicability as far as engineering problems were concerned. In our present study we wish to approach the problem of applicability from another angle. The conditions for large-scale production had to be worked out, the problem of crust formation solved and, last but not least, reliable data had to be collected for the calculation of economic parameters before large-scale introduction of the process could be envisaged.

In these experiments a dough prepared in the Research Institute of the Bakery Industry (SKI) was used. The composition of the dough corresponded more or less to that of half-brown bread, it was prepared of wheat flour BL 80 and rye flour RL 90. This is a somewhat more complex composition than the one used in laboratory experiments containing wheat flour only, but no difficulties were encountered in its processing in the dielectric field.

The technology applied to the production of bread divides the heat transfer process into two stages. Up to a temperature of about 80 to 85 °C the leavened dough was heated in the high frequency dielectric field, while the last step of baking, including crust formation, was performed in a baking oven, used in traditional technologies. By means of this combined procedure an appearance of the product was achieved which conformed to the taste of the consumer, while the texture of the crumb possessed all the advantages resulting from the application of the dielectric field. By means of this technology a certain, though slight, saving in materials could be achieved with simultaneous decrease in production cost.

In the course of the last twenty years an increasing number of reports of an informative nature has been published, mainly in American and West European trade journals and books, on the application of dielectric heat transfer in the baking industry, reflecting the favourable reception of the process by the industry.

In countries in which electrical energy is available in large quantity at low cost, researchers have tried to find solutions based fully or almost fully on the utilization of electric power. This, of course, is also true for technologies which, besides dielectric heat transfer, use some external radiating heat source,

primarily in the interest of obtaining the desired outer appearance of the product. Such methods are those described by HOLLAND (1963), MANWARING (1967) and HAFNER (1968) which combine dielectric heat transfer with IR radiation technique. A new trend is reflected in the report of the Chorleywood Flour Milling and Baking Research Association (ANON, 1971) where attempts were made to combine microwave engineering with traditional baking technology. The greatest problem here was caused by the limited penetration power of microwave energy due to which only magnetrons operating at shorter wavelengths could be successfully used, but even then only products of limited thickness could be processed. Other systems utilizing microwave techniques have the same drawback of being able to bake only comparatively thin doughs (BOORAS & KEENE, 1968; BALDWIN *et al.*, 1968; DECAREAU, 1970).

Energetics and energy costs in Hungary give a definite outline to the conditions by which the problem of applicability can be approached from the economic aspect. Application of electrical energy should be limited to the absolute minimum necessitated by the technological procedure, that is, only if it is associated with some definite advantage arising directly from the new procedure. Hungarian consumer requirements are characterized by the public's insistence on the traditional appearance of its bread, meaning that the new technology must ensure a loose texture of the crumb surrounded by a crust of the characteristic colour.

Bearing all these points in mind, we have designed our experiments with the aim of solving the problem with the successive application of the high frequency dielectric procedure and of the baking oven known by the Hungarian baking industry as the one with the most favourable characteristics.

The results of laboratory experiments have confirmed the suitability of high frequency dielectric heat transfer chosen for the complete and uniform warming of the dough. They have also shown that characteristic structural changes will take place in the bread already at temperatures between 75 and 80°C, thus it is absolutely necessary to apply dielectric heat transfer up to this temperature.

Application of the traditional baking method seemed most appropriate in the last stage of the baking process, partly because the energy needed in this finishing stage will involve the lowest cost and partly because in this way the most up-to-date continuous-operation tunnel ovens of the baking plants can still be used. In case of implementation on the industrial scale, the two continuously operating equipments ought to be connected through an equally continuously operating device ensuring vibration- free transfer of the product from one line to the other.

Comparison of the currently available and operating baking ovens in Hungary has shown that the most suitable for our purpose is the continuously operating baking tunnel Mecatherm Transvap type II. 20 which will shortly

be produced on a large scale in Hungary. The construction of the oven is based on a French Patent, it is oil-heated and has an output of 500 kg per hour (MAKÁRY, 1971).

In our Institute the temperature conditions prevailing in a Mecatherm tunnel during baking, could be reproduced by means of this equipment, and with the help of a drying cabinet.

1. Materials and methods

1.1. Dough

The dough for the experiments was obtained by courtesy of SKI (Research Institute of the Bakery Industry, Budapest). The composition of the dough for 100 kg of bread was:

62.00 kg wheat flour BL 80
11.00 kg rye flour RL 90
1.10 kg dried yeast
0.75 kg sugar
1.50 kg salt
0.45 kg polyacid
42.00 kg water.

The quality of the wheat flour BL 80 complied with the specifications of Hungarian Standard MSZ 6336—70, that of the rye flour RL 90 with MÉMSZ Standard 1351 (BL stands for wheat flour, RL for rye flour, 80 and 90, resp., mean that the ash content of the flour is 0.80 and 0.90%, resp. of the solids content).

1.2. Baking equipment and instruments

1.2.1. High frequency generator used in the first stage of baking and dough conveyor device. The leavened dough was heated to 80 to 85°C in the dielectric field of the Institute's Brown-Boveri type C 10 high frequency dielectric generator. This generator, together with the work capacitor connected to the outer oscillating circuit of the generator for the sake of output control, are shown in Fig. 1.

The work capacitor was surrounded by a closed aluminium shield which could be opened after the current had been switched off. On the generator side along the entire length of the shield a copper wire netting ensured the continuous control of the manipulating area. This protective netting was removed before taking the photographs in order to make the details more clearly visible. The capacitor plates in vertical and parallel arrangement were fixed to the protective network as shown in Fig. 2.

The leavened dough is filled into polyethylene containers of a base of 240 ×150 mm, a height of 100 mm and sides of 150 ×100 mm. The containers are placed side by side between the plates of the work capacitor and slide along horizontal polymethacrylate rods whose axes are parallel to the work capacitor. To avoid direct contact with the polyethylene containers an about 20 mm wide Teflon conducting strip was fixed to the internal plates of the capacitor.

The device for conveying the leavened dough in the polyethylene containers is shown in Fig. 3.

The prismatic shape of the polyethylene containers greatly facilitated simple and accurate measurement under laboratory conditions. Under plant conditions forms other than prismatic, conforming both to the taste of the customer and to the demands of expediency, would present no problem.

The asynchronous motor fixed to the scaffolding is connected through a belt transmission with the two-stage driving work which operates the sliding elements for the transport of the containers through a chain-drive. A sensing and control switch in the way of the conveyed containers provides for the exact distance of one container-length between two containers. This discontinuous conveyance which does not interfere with the continuous character of the operation had to be applied to avoid the addition of an extremely costly second driving gear.

The temperature of the dough between the plates of the work capacitor was checked by means of a toluene-filled thermometer placed directly into the dough.

The overall electric power consumption of the high frequency generator was measured with a ferrodynamic outputmeter type RFWd (manufactured by Ganz Instrument Works EKM Budapest). This outputmeter was provided with a recording device and connected through a reductor to the high voltage supply line of the generator.

1.2.2. Oven for crust formation. The Institute possesses an electrically heated, continuously variable temperature controlled oven, shown in Fig. 4.

The process controlling temperature regulator and the ammeters indicating current uptake can be seen on the instrument panel.

2. Results

In our earlier paper we have already reported on the characteristics of bread prepared in the high frequency dielectric field and on its comparison with bread prepared by traditional technology (RUNTÁG & DEMECZKY, 1973). In our present work we have set ourselves the target of constructing an equipment of continuous operation, as well as of determining the conditions of crust formation. A further objective was to establish, on the ground of experimental

results, the economic factors of the application of high frequency heat transfer in the baking industry, particularly in bread baking.

The results of the experiments have confirmed the suitability of the combined technology for baking bread to form the basis of a manufacturing process under industrial conditions.

Beyond working out the continuously operating equipment, we consider as the most important part of our work the fact that in course of our combined baking experiments we were able to establish that the new technology advantageously unites, with respect to both manufacturing conditions and the quality of the product, all favourable features of the two processes thereby ensuring the competitiveness of the new combined technology.

The desired texture of the crumb and a volume 20 to 25% greater than the one obtained by traditional baking technology can be achieved by means of the application of the dielectric field already at temperatures as low as 80 to 85°C. The other phases of the heat treatment process: maintenance of the temperature, provision for the removal of moisture and formation of the crust by way of the traditional technological methods can be achieved with energy carriers cheaper than electric power. The product obtained by the combined baking technique is shown in Fig. 5.

These experiments have convinced us that the transfer of the dough from the dielectric to the traditional baking phase, involving some slight disturbance of the operation, can be performed without danger to quality when the texture has not only developed, but has already set to a certain degree. This phenomenon associated with technological reasons justified the fact that, as a result of our work, we have not assigned a temperature of 75 to 80°C at which the texture of the crumb develops to the final phase of dielectric heat transfer, but envisage a temperature range of 80 to 85°C at which the structure of the crumb sets.

From our experimental results we have arrived to the conclusion that under the given capacitor conditions and with the available generator the heating of the dough to 80° to 85°C is a process with a specific power requirement of 0.16 to 0.18 kWh per kg of dough, which means that electric power is only needed up to 55 to 60% of the total heat requirement of baking. It must, however, be ensured that after treatment in the dielectric field the dough is transferred within the shortest possible time to the oven where the second phase of baking will take place.

We have further found that, by providing a temperature of 250 to 280°C, baking can be completed with satisfactory safety and giving a high quality product in the oven at our disposal in 6 to 8 minutes. The thickness, colour and uniformity of the crust will satisfy even the most exacting customer. We believe that for the performance of the after-baking procedure the complex construction of the oven types in the plants can be considerably simplified.

The device for blowing steam into the oven, usually applied in bakery ovens, becomes superfluous, since there is no longer any danger of a too dry, desiccated crust. In the combined baking technology the product is placed into the oven in the stage when moisture removal is the most intensive. In contrast to the traditional technology, where steam has to be introduced from the outside and then removed by additional energy input, in the combined technology the product itself provides the necessary steam.

According to our measurements a saving in weight can be achieved by means of the combined method, primarily by reducing the heat load on the layers near the crust. The fact that the heat needed for the development of the texture of the crumb is not transferred through the already formed crust of poor heat conductivity has not only a favourable influence on the heat balance of the process, but also by eliminating the excessive and unnecessary desiccation of the outer layers, that is, by reducing the loss of material in baking, advantageously influences the economy of the process. Our tentative measurements have shown that the technology worked out by us reduces the weight loss of baking by about 14% compared to the loss encountered with traditional technologies.

3. Conclusions

Both laboratory and pilot plant experiments have furnished convincing evidence on the applicability of the combination of high frequency and traditional technologies in the form of continuous operations to the production of bread of a quality superior to that produced by traditional methods. Thanks to the new technology, production is realized under conditions more favourable and not less efficient economically than by traditional baking method.

On the basis of these experiments the technological parameters of a line in which 1 000 kg per hour output high frequency operation was combined with traditional baking was compared to those of an actually operating industrial line having at present the best technical and economic parameters.

The most important technological and economic parameters forming the basis of this comparison are summarized in Table 1.

Hungarian bakeries use at present a 1 000 kg per hour output tunnel oven, type PTC-52/260 manufactured in Poland. The oven is provided with a cyclotherm heating system, operates continuously and is used for traditional baking. Four workers operate the almost 24 m long and 2.5 m wide equipment which uses three types of energy carriers: its oil consumption is 52.5 kg h^{-1}, its electric power requirement 23 kWh and in addition it needs 210 kg of steam of 0.3 gauge pressure.

For the combined baking technology two Brown-Boveri high frequency generators, type C 50, each with 25 kWh useful output and a Mecatherm Transvap type II.20 tunnel oven with 500 kg h^{-1} output were chosen. This

Table 1

Main technological parameters of the baking lines of 1 000 kg h^{-1} output and the effect of their operation on cost

Baking method	Type of equipment, floor space, m^2	A	B		C	D	E
			a	b			
Traditional	PTC-52/250; 60	2 200	52.50	23.00	46	—	26.79
Combined	2 Brown Boveri generators, type C-50, 1 Mecatherm tunnel oven; 28	3 700	14.00	161.70	18	2.00	22.11
High frequency crustless	one type C 100 and one type C 50 Brown Boveri generator; 15	2 000	—	320.00	18	2.00	22.91

A: Purchasing price in 1 000 Forints (Ft)
B: Power requirement.
a: Fuel oil, kg h^{-1}.
b: Electric power, kWh^{-1}.
C: Wages, Ft h^{-1}.
D: Material saving, % (w/w).
E: Specific operation cost, Ft per 100 kg of bread.

equipment occupies altogether 28 m^2 floor space and is operated by two workers. This line consumes 162 kWh electric power and 14 kg h^{-1} of fuel oil, so that it reduces the cost of the operation — bearing in mind the lower loss in weight — by about 15 to 20 per cent, or 4 Hungarian Forints (Ft) per 100 kg of product.

Finally, we worked out the probable economic parameters of a baking line operated solely by high frequency energy. The product of this line will have no crust, so that some other operation will be needed for crust formation, or the bread will have to be protected by special packaging from environmental injuries.

For the operation of the line working exclusively by high frequency energy, a type C 100 and a type C 50 Brown-Boveri generators were planned. This dielectric equipment of a 150 kWh overall useful output has a floor space requirement of not more than 15 m^2, with the additional advantage of requiring a single energy carrier. Due, however, to the specific electric power requirement of 300 to 320 kWh and primarily to the high consumer price of electric power in Hungary, it does not seem likely that the method will be applied in the near future in Hungarian bakeries. The saving in material which can be achieved by the new technology is balanced by the current high price of packing.

*

We wish to thank Dr. L. SZALAI, director and Mrs. J. BOGDÁN, head of department, both of SKI who with their understanding of our practical problems greatly facilitated our work. Thanks are also due to Mr. J. OFFENBECK, Central Food Research Institute, for his devoted help in the performance of the experiments.

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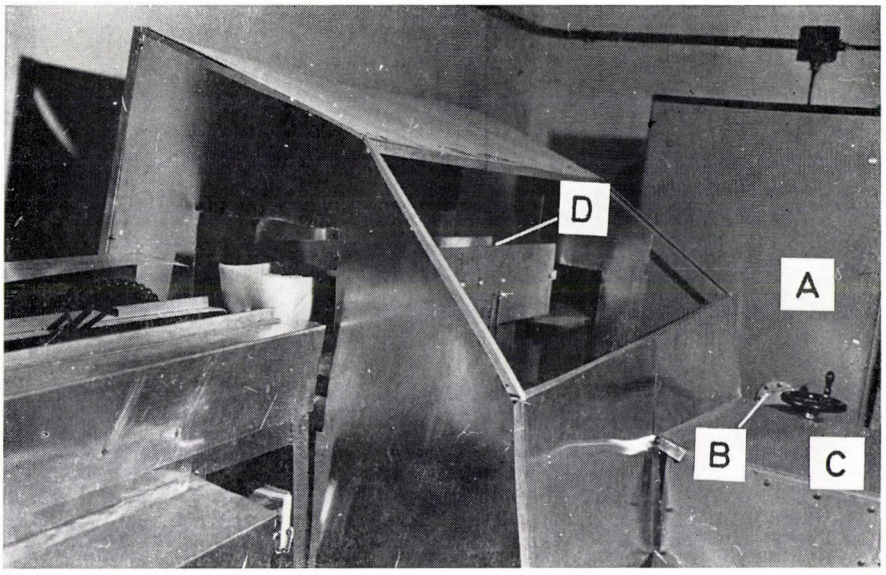

Fig. 1. Dielectric high frequency generator and its external oscillating circuit. A — high frequency generator type C 10; B — coaxial cable; C — tuning inductivity; D — plates of work capacitor

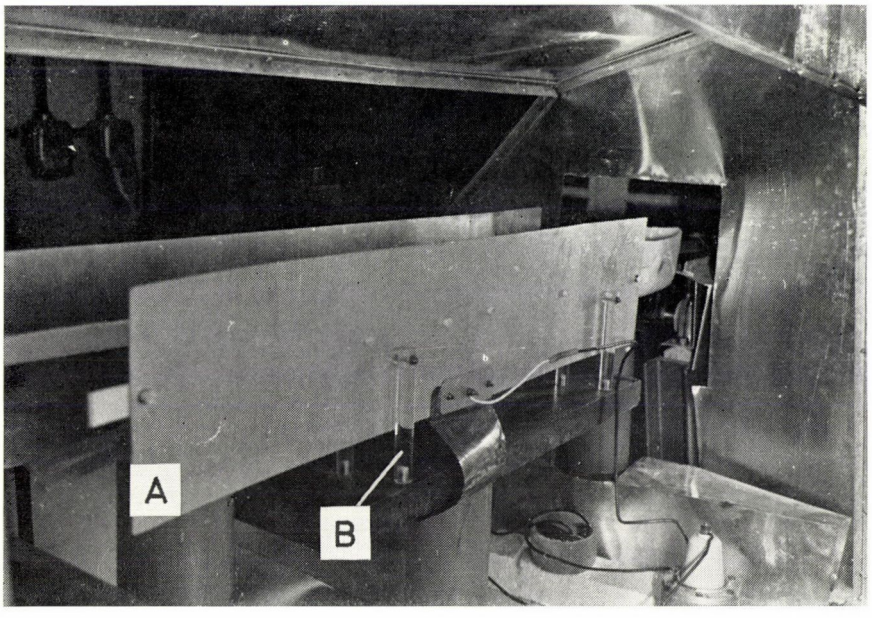

Fig. 2. Arrangement of the work capacitor plates in the high frequency field. A — plates of work capacitor; B — supporting rods

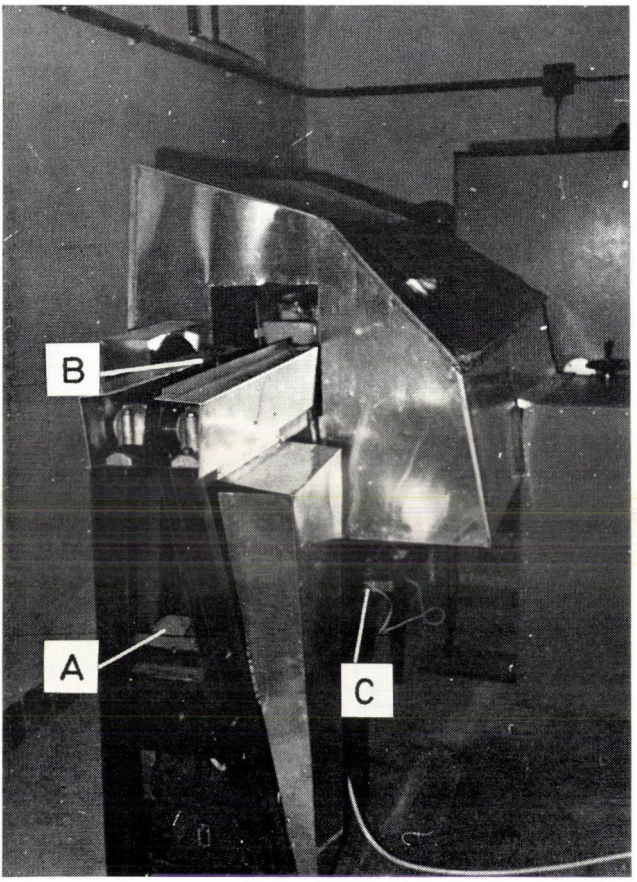

Fig. 3. Feeding device. A — driving motor; B — conveying elements; C — control switch

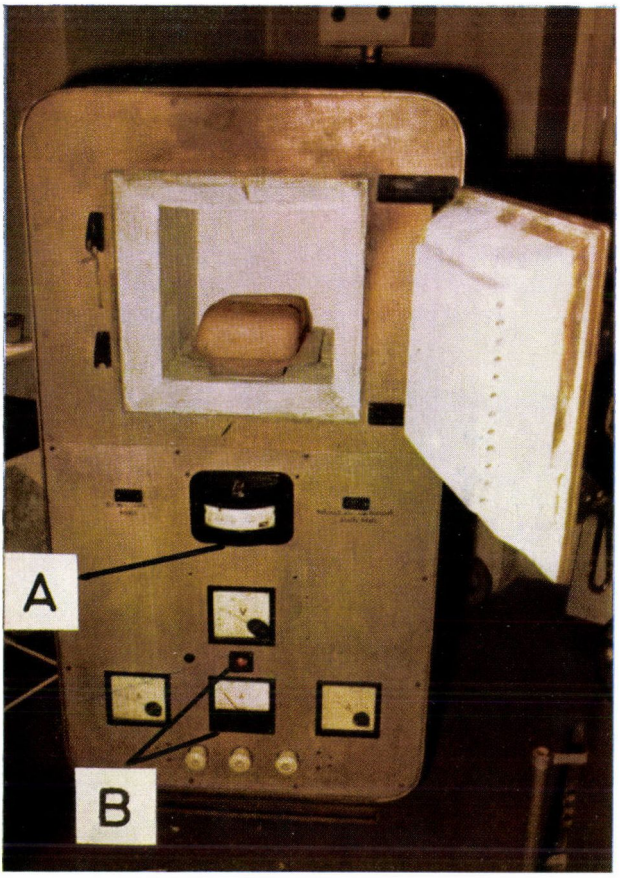

Fig. 4. Electrically heated oven. A — temperature regulator; B — outputmeter

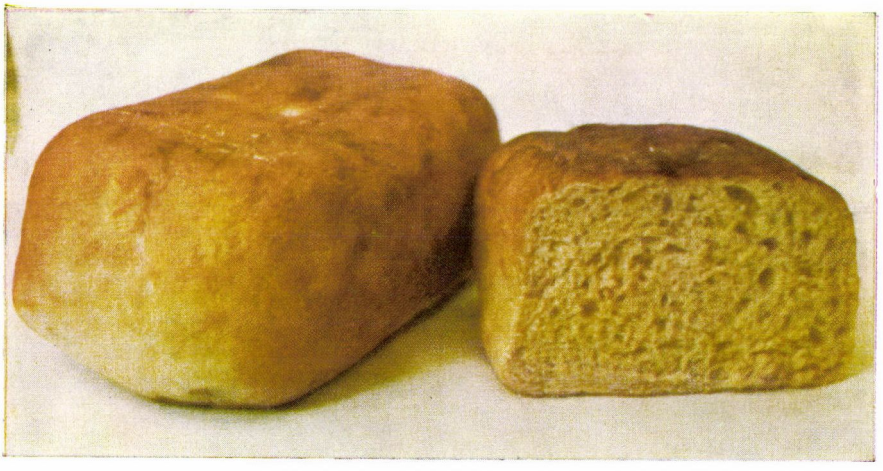

Fig. 5. Bread produced by means of the combined baking method

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STUDIES ON VEGETABLE OIL HARDENING TECHNOLOGIES BY MEANS OF THE ANALYTICAL PARAMETERS OF FATS WITH SPECIAL REFERENCE TO THE FORMATION OF TRANS ISOMER FATTY ACIDS

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The technology of commercial-scale hydrogenation of soya-bean oil–rape-seed oil mixtures and of sunflower-seed oil in Hungarian factories was subjected to investigation. On the basis of the analytical parameters the process of hydrogenation can be divided into two stages, the first stage being characterized by the conversion of linoleic acid into oleic acid and an intensive trans isomer formation. Thus, in stage one the increase in slip point is caused primarily by intensive oleic acid and trans isomer formation, so that, essentially, this stage can be considered as a selective one. In the second stage the formation of stearic acid is more intensive than in the first stage, so that the increase in slip point can in fact be attributed to the formation of stearic acid. Consequently this second stage has an inselective character.

A comparison was made between the analytical data of the typically selective and inselective commercial-scale hydrogenations of sunflower-seed oils hardened to identical slip points. The results have indicated that in case of inselective hydrogenation the hardening period will be shorter and less trans isomer and more stearic acid will be formed. The formation of stearic acid runs parallel to the linoleic acid → oleic acid conversion. The higher slip point value and the lower content in trans isomers suggest a difference in the triglyceride structure.

In agreement with literature data it was found that trans isomer formation occurs simultaneously with the linoleic acid → oleic acid transformation and that at higher temperatures (150 to 180 °C) more oleic acid and trans fatty acids are formed during unit time.

At the beginning of hardening the trans isomer content is divided between oleic and linoleic acid in a ratio of 1 : 1, while when maximum trans isomer content has been reached this ratio shifts in the direction of 3 : 1.

The rise in margarine consumption in Hungary has given an impetus to the production of a commodity with taste and consistency similar to that of butter, while having more favourable physiological effects than the latter. One of the conditions is an up-to-date hardening technology by means of which the industry can prepare base materials of adequate quality.

For the production of margarine base materials, in the past the hardening of the fatty substances was directed to ensure the formation of the highest possible percentage of fatty acid isomers, with relatively high iodine numbers besides high slip points (NIELSEN *et al.*, 1960). Owing to the difference between the melting points of the fatty acid isomers, and the different energy content of the molecules, two fats may have the same iodine number — expressing the unsaturation of the molecule — but different slip points and different tendencies to crystallization. Thus, the formation of the iso-acids has a marked influ-

ence on consistency. The presence of trans fatty acids was found definitely advantageous in margarine production, since these are easier to emulsify and possess in addition more favourable crystallization properties than fat base materials with the same slip point, but containing more stearic acid.

In recent years those engaged in margarine production had to face a dual task. Beside the desired consistency, characterized by significantly lower trans fatty acid content than earlier, sufficient essential fatty acid content is also an indispensable condition of high quality. These requirements can be met by preparing the fat base of margarine from several basic fats of differing degrees of hydrogenation.

Consequently, the hardening technologies of the basic fats of margarine may also be different (ALLEN, 1960). Two fundamental methods of catalytic hydrogenation of oils are known:

a) a sequence of reactions of selective nature, characterized by a starting temperature of 160—180°C, hydrogenation at atmospheric pressure in the presence of a catalyst of medium activity;

b) a sequence of reactions of inselective nature characterized by a lower starting temperature of 100—120°C, hydrogenation at 3 to 7 gauge pressure, in the presence of fresh catalyst under intensive agitation.

This second way of hydrogenation is of special importance for oils containing poly-unsaturated fatty acids, since under these conditions the formation of undesirable heterocyclic compounds is hindered. The current up-to-date technologies were evolved from the different combinations of the two trends (OSTEROTH, 1970).

We studied the changes in the chemical parameters of the basic fats hardened by different procedures. Our experiments were aimed at the determination by analytical methods of the factors causing a rise in the slip points of hardened fats (trans fatty acids, stearic acid) and at establishing from the experimental results the character of the hydrogenation reaction taking place under given plant conditions.

1. Materials and methods

1.1. Materials

The commercial scale hydrogenation of soya-bean and rape-seed oil mixtures and of sunflower-seed oil was studied. The samples came from batch-type hardening plant operating according to the Normann technology, and using Cu–Ni catalyst with 20 to 25 % Ni-content.

The studies were extended to follow up a plant scale hydrogenation process in which the parameters are modified to favour the inselective type of hardening. In this case sunflower-seed oil was hydrogenated. Hardening was performed at a relatively low temperature (104 to 160°C) at 0.7 gauge pressure.

Samples were taken every 30 minutes. The slip point, fatty acid composition and isolated trans fatty acid quantity of the samples were determined as functions of the hardening time and of temperature. The distribution during hardening of trans fatty acids between fatty acids $C_{18:1}$ and $C_{18:2}$ of selectively hydrogenated sunflower-seed oil was also determined.

Three parallel laboratory experiments were performed and the data in the Tables are the averages of the parallel results.

1.2. Analytical procedures

1.2.1. Determination of slip point. The slip points of the samples were determined according to Hungarian Standard No. 19821—53 with an electric slip-point-meter.

1.2.2. Determination of fatty acid composition. The fatty acid composition was determined with a Pye-Unicam type gas chromatograph. The experimental conditions were:

length of column	1.8 m
internal diameter of column	0.4 m
type of support	Chromosorb W
particle size	110—120 mesh
type of stationary phase	ethyleneglycol-succinate
concentration	10%
temperature: thermostat	188°C
detector	245°C
evaporator	240°C
detector	flame ionization
carrier gas	nitrogen
flow rate	45—50 ml · min^{-1}

The gas chromatograms were plotted from the methyl esters of the samples. For the preparation of the methyl esters the method of CHALVARDJIAN and co-workers was used (1964).

1.2.3. Quantitative determination of isolated trans fatty acids. The determination was performed with the infra-red spectrophotometer, type Zeiss UR 20 according to the 1970 IUPAC recommendation (A.O.C.S., 1970).

In case of methyl esters the maximum of the band originating from the C—H deformation oscillation of the trans double bond appears in the infra-red spectrum at a wave number of 965 cm^{-1}. The corresponding cis and saturated systems contain no similar absorption in their spectra.

The methyl esters prepared from the samples were purified by thin-layer chromatography (MALINS & MANGOLD, 1960). The infra-red spectra were

recorded from the diluted solutions of known concentrations of the purified methyl esters in the 900 to 1 100 cm^{-1} range. The percentage isolated trans isomer was calculated from the calibration curve previously obtained from methyl elaidate. Thus, percentage trans isomer is a relative value giving the per cent of trans fatty acids, expressed in the form of methyl elaidate, in the methyl ester of the fat.

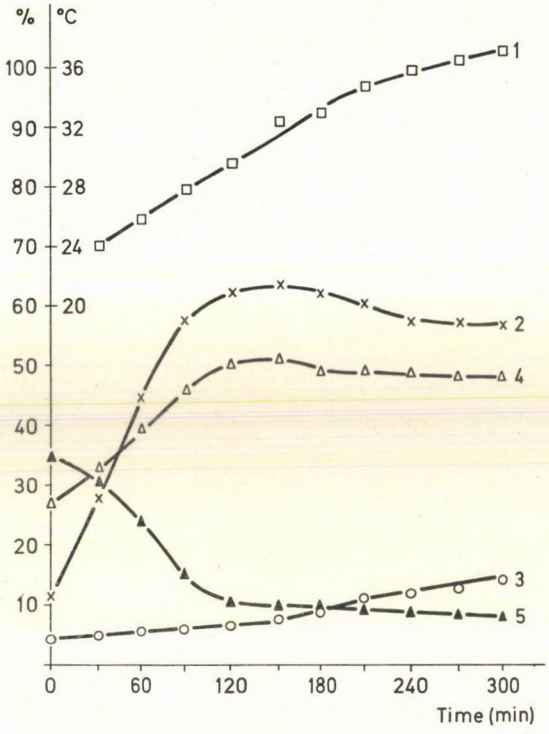

Fig. 1. Analytical parameters of a selectively hydrogenated soya-bean–rape-seed oil mixture *vs.* time (from the data in Table 1). 1 — slip point; 2 — i-trans fatty acid; 3 — stearic acid; 4 — oleic acid; 5 — linoleic acid

1.2.4. Determination of the distribution of isolated trans content between monoene and polyene fatty acids. The methyl esters were separated by thin-layer chromatography on silver nitrate impregnated Kieselgel G adsorbent on the basis of their saturation (MORRIS, 1966). The silver ions from π-complexes with the double bonds and the stability and distribution of the complex on the layer depend on the number of double bonds and on geometrical configuration. The separation of the saturated monoene and diene fractions on the layer was checked by gas chromatography. Separation resulted in fractions of at least 95% purity. The trans content of the monoene fraction was

determined according to the method described in para. 1.2.3. The trans content of the diene fraction was calculated from the difference between the total trans content and the trans content of the monoene fraction.

Table 1

Analytical data of a selectively hardened soya-bean–rape-seed oil mixture

Time, minute	Hardening temperature, °C	Slip point, °C	Isolated trans isomer fatty acid, %	Fatty acid composition, %								
				$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{20:1}$	$C_{22:0}$	$C_{22:1}$
0	140	—	11.4	7.0	4.1	26.8	34.4	5.5	1.4	3.1	0.5	18.0
30	164	24.0	27.6	7.1	4.8	32.6	31.0	2.6	1.0	3.2	0.4	17.3
60	180	25.9	44.5	7.0	5.5	39.6	23.8	1.1	1.2	3.3	0.6	17.9
90	190	26.9	57.2	8.0	5.8	46.0	15.2	—	1.3	3.3	0.5	19.7
120	198	29.6	61.8	8.2	6.4	50.0	10.1	—	1.4	3.2	0.9	19.8
150	204	32.4	63.5	7.8	7.4	51.0	10.0	—	1.2	3.1	0.8	18.7
180	206	33.1	61.7	7.9	9.1	49.0	9.5	—	1.4	3.2	1.0	18.9
210	206	34.9	60.2	8.0	11.1	49.0	9.2	—	1.3	3.2	1.2	17.0
240	206	35.8	57.3	8.1	11.8	48.5	8.5	—	1.1	3.3	1.2	18.5
270	206	36.5	57.4	7.9	12.5	48.0	8.0	—	1.6	3.0	1.4	17.6
300	206	37.2	56.5	7.0	14.2	48.0	7.6	—	1.4	3.0	1.5	17.3

Composition of the mixture: 60% soya-bean oil and 40% rape-seed oil,
quantity: 7 500 kg.
Flow rate of hydrogen: 140 m^3 per hour.
Catalyst: Cu—Ni with 20 to 25% Ni content; quantity: 75 kg of fresh catalyst and 75 kg of a catalyst once used in the hardening process.

2. Results

2.1. Selective type hardening of a soya-bean and rape-seed oil mixture

Table 1 contains the analytical parameters determined by us for a selectively hardened soya-bean and rape-seed oil mixture. Fig. 1 shows the changes in the analytical parameters of Table 1 *vs.* hardening time and temperature.

The mixture was hardened for 300 minutes when the endproduct had a slip point of 37°C. It appears from Fig. 1 that similarly to the formation of oleic acid, the formation of the trans fatty acid isomer gives a curve with a maximum. The decrease in linoleic acid is proportional to the rise in the quantity of oleic acid. During hardening the slip point increases monotonously.

2.2. Selectively hardened sunflower-seed oil

The experimental results are given in Table 2 and in Fig. 2. The oil was hardened for 180 minutes when the fat obtained as endproduct had a slip point of 29°C.

On plotting the analytical parameters of sunflower-seed oil against the hardening period and temperature, a curve similar to that of the soya-bean –rape-seed oil mixture is obtained.

Table 2

Analytical data of selectively hardened sunflower oil

Time, minute	Hardening temperature, °C	Slip point, °C	Isolated trans isomer fatty acid, %	Fatty acid composition, %			
				$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
0	84	—	10.4	6.5	5.1	30.3	58.1
30	108	—	16.2	6.7	6.1	33.3	53.9
60	118	—	25.3	7.6	5.9	36.2	50.3
90	144	22.5	41.9	7.2	7.0	45.6	40.2
120	178	25.2	51.0	7.2	7.5	56.1	29.2
150	186	27.2	58.8	7.0	8.5	69.1	15.4
180	180	29.1	62.4	6.8	8.9	72.0	12.3

Sunflower-seed oil quantity: 7 500 kg.
Hydrogen flow rate: 140 m³ per hour.
Catalyst: Cu–Ni with 20 to 25% of Ni content; quantity: 75 kg of fresh catalyst and 75 kg of a catalyst once used for hardening.

2.3. Sunflower-seed oil hardened under inselective conditions

Changes of the pertaining analytical parameters *vs.* time and temperature are given in Table 3 and Fig. 2.

The oil was hardened for 90 minutes when a fatty endproduct with a slip point of 29°C was obtained. The formation of stearic acid takes place simultaneously with the linoleic acid→oleic acid conversion, at the same time the rate of trans isomer formation is characterized by a maximum curve which is less steep than that of oleic acid.

2.4. Temperature dependence of the formation of isolated trans isomer fatty acids

The formation of trans fatty acids as a function of in-plant hardening temperature was investigated in case of the soya-bean and rape-seed oil mixture. The results are shown in Table 4. The percentage of trans isomer *vs.* temperature curves for plant scale hardenings at different temperatures are given in Fig. 3.

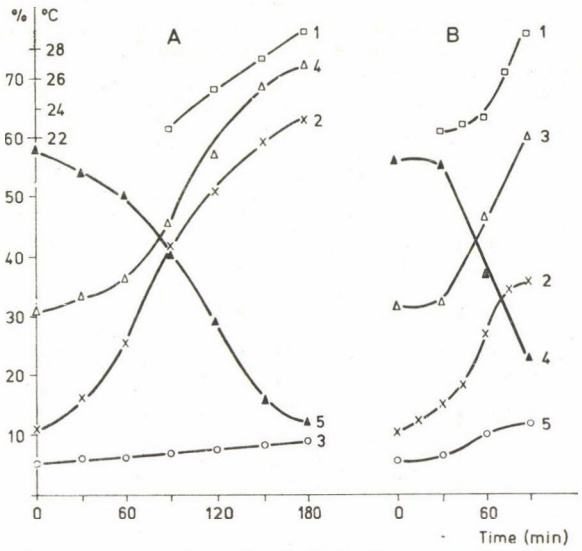

Fig. 2. A: Analytical parameters of a selectively hydrogenated sunflower-seed oil *vs.* time (from the data in Table 2). B: Analytical parameters of inselectively hydrogenated sunflower-seed oil *vs.* time (from the data in Table 3). 1 — Slip point; 2 — i-trans fatty acid; 3 — stearic acid; 4 — oleic acid; 5 – linoleic acid

Table 3

Analytical data of inselectively hardened sunflower-seed oil

Time, minute	Hardening temperature, °C	Slip point, °C	Isolated trans isomer fatty acid, %	Fatty acid composition, %			
				C_{18}:0	C_{18}:0	C_{18}:1	C_{18}:2
0	104	—	9.8	6.2	5.7	31.6	56.5
15	120	—	12.2				
30	124	22.2	15.0	6.3	6.5	32.0	55.2
45	132	22.8	18.0				
60	142	23.2	27.0	6.5	9.7	47.4	36.4
75	147	26.3	34.0				
90	160	28.9	35.6	6.8	10.5	60.2	22.5

Sunflower-seed oil quantity: 7 500 kg.
Hydrogen flow rate: 140 m³ per hour.
Catalyst: Cu—Ni with 20 to 25% of nickel content, quantity: 100 kg of fresh catalyst.

Table 4

Trans fatty acid content of the soya-bean–rape-seed oil mixture vs. temperature

Time, minute	Hardening at plant							
	1		2		3		4	
	A	B	A	B	A	B	A	B
0	106	11.0	140	11.4	165	8.3	172	11.3
30	120	11.0	164	27.6	176	28.6	180	29.3
60	136	15.6	180	44.5	186	42.0	188	44.1
90	152	22.4	190	57.2	196	58.5	198	55.8
120	168	35.9	198	61.8	206	60.3	206	62.8
150	182	47.4	204	63.5				
180	186	53.2						
210	192	59.9						
240	198	65.0						

A = temperature, °C.
B = trans fatty acid content, %.

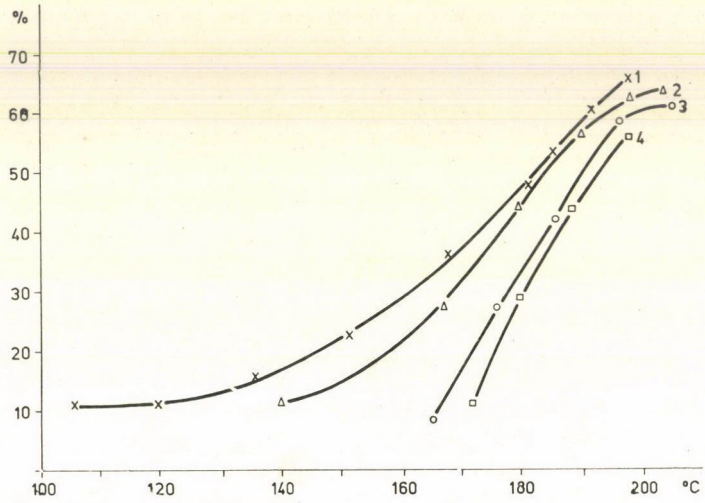

Fig. 3. Changes in the trans fatty acid content of a soya-bean–rape-seed oil mixture hardened with different starting temperatures *vs.* temperature (from the data in Table 4). 1 — 1st in-plant hardening; 2 — 2nd in-plant hardening; 3 — 3nd in-plant hardening; 4 — 4th in-plant hardening

2.5. Quantitative distribution of isolated trans isomer fatty acids between fatty acid fractions $C_{18:1}$ and $C_{18:2}$

The distribution tests were carried out on samples of sunflower-seed oil (para. 2.2). The samples were taken every 30 minutes and the results are shown in Table 5 and Fig. 4.

Table 5

Distribution of the isolated trans isomer fatty acid content between fatty acids $C_{18:1}$ and $C_{18:2}$ in selectively hydrogenated sunflower-seed oil

Time, minute	Total trans content, %	trans $C_{18:1}$ fraction, %		trans $C_{18:2}$ fraction, %	
		a	b	a	b
0	10.4	5.5	18.3	4.9	8.5
30	16.2	8.0	24.0	8.2	15.2
60	25.3	12.3	34.0	13.0	24.4
90	41.9	22.5	49.4	19.4	48.3
120	51.0	32.2	57.5	18.8	64.5
150	58.8	42.9	62.2	15.9	103.0
180	62.4	46.0	63.9	16.4	133.0

a = % of sunflower-seed oil.
b = % of fractions $C_{18:1}$ and $C_{18:2}$, resp.

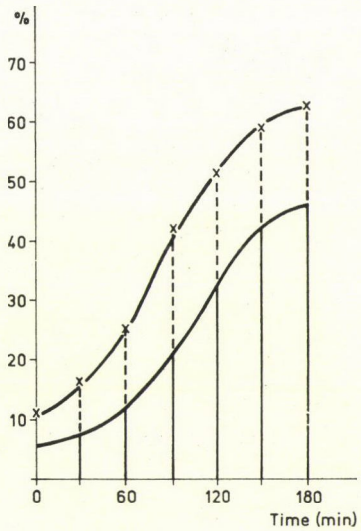

Fig. 4. Distribution of the quantity of trans fatty acids between the $C_{18:1}$ and $C_{18:2}$ fatty acids of selectively hardened sunflower-seed oil (data taken from Table 5). — x — Quantity of trans fatty acids; ——— trans fatty acid content of fraction $C_{18:1}$; – – – – trans fatty acid content of fraction $C_{18:2}$

3. Conclusions

3.1. Selectively hardened vegetable oils

According to the results, in the hardening of the soya-bean–rape-seed oil mixture two phases may be distinguished.

Table 6

a) *Changes in the main analytical parameters of the soya-bean–rape-seed oil mixture in the two stages of the hardening process*

Analytical parameters	1st stage	2nd stage	Total
Slip point, °C	5.6	7.6	13.2
Trans isomer fatty acid, %	50.4	5.3	55.3
$C_{18:2}$, %	24.3	2.5	26.8
$C_{18:1}$, %	23.2	3.0	26.2
$C_{18:0}$, %	2.3	7.8	10.1

b) *Percentage distribution of changes in analytical parameters between the two stages of hardening*

Analytical parameters	1st stage	2nd stage	Total
Slip point, °C	42.5	57.5	100.0
Trans isomer fatty acid, %	89.4	10.6	100.0
$C_{18:2}$, %	90.6	9.4	100.0
$C_{18:1}$, %	89.4	10.6	100.0
$C_{18:0}$, %	22.7	77.3	100.0

The first phase lasts from 0 to 120 min when a slip point of 29.6°C is reached. This phase is characterized by the linoleic acid → oleic acid conversion and a simultaneous intensive trans isomer formation. The trans content increases from 11 to 62%. The rise in slip point is caused primarily by the conversion of linoleic acid and by isomer formation. The amount of stearic acid formed is negligible, hence this phase is typically a selective one.

In the second phase which is associated with a rise of the slip point from 29.6° to 37.2°C the formation of stearic acid is more intensive and the overall quantity of oleic and linoleic acids decreases proportionally. The trans isomer content also shows a slightly decreasing tendency, thus the formation of stearic acid is primarily responsible for the higher slip point, consequently this phase of the hardening process is characterized by inselectivity.

The analytical parameters of the sunflower-seed oil, selectively hardened up to a slip point of 30°C, show similar changes as those observed in the first phase of the hardening of the soya-bean–rape-seed oil mixture. The rise in slip point is affected mainly by the formation of oleic acid and its isomers, hence hardening can be considered selective.

3.2. Sunflower-seed oil hardened under inselective conditions

Changes in the analytical parameters of sunflower-seed oil hardened under selective and inselective conditions up to a slip point of 29°C are shown

in Fig. 2. There is a marked difference in hardening time when sunflower-seed oil is hardened in two different ways up to the same slip point: the time for selective hardening is 180 min, that for inselective hardening 90 min. During this period almost twice the amount of trans isomer is formed by selective hardening than by the inselective procedure, and at the same time the linoleic acid content of the endproduct is 10% higher when hardening is performed under inselective conditions.

If the endproduct of inselective hardening is compared to the intermediary product of selective hardening with identical linoleic acid → oleic acid conversion, it appears that in case of inselective hardening the slip point is 2.9 units higher, the quantity of trans isomers 19.4% less and the quantity of stearic acid 3.5% higher than in case of selective hardening.

Table 7

Comparison of selective and inselective hardening

Analytical parameters	Selective hardening	Inselective hardening	Difference
Time, minute	135	90	45
Temperature, °C	182	160	22
Slip point, °C	26	28.9	2.9
Trans fatty acid, %	55	35.6	19.4
$C_{18:2}$, %	22.5	22.5	0
$C_{18:1}$, %	60.2	60.2	0
$C_{18:0}$, %	8.0	11.5	3.5

These findings suggest a significant difference between the triglyceride structures of the two hardened substances, which by the possible formation of tri- or di-saturated groups explains on the basis of the analytical parameters the fact that a product of higher slip point is formed in the presence of considerably lower quantities of the isomer and only a few per cent more of stearic acid.

3.3. Temperature dependence of the formation of isolated trans isomer fatty acids

In agreement with literature data our experimental results confirm the temperature dependence of hardening. Linoleic acid → oleic acid conversion proceeds parallel to the formation of the trans isomer in the same temperature range and with similar high intensity. Between 100° and 150 °C the formation of the trans isomer is a considerably slower process than above 150°C. If

hydrogenation starts at a higher temperature, more trans isomers will be formed in unit time and the linoleic acid → oleic acid conversion will also proceed at a higher rate, consequently the first phase of hardening will be completed within a shorter period of time. When hardening starts at 105°C, 3.5 hours are needed for obtaining a fat with a slip point of 30°C, while if hardening starts at 140°C a product with the same slip point as before is formed in 2.5 hours.

3.4. Quantitative distribution of the trans isomer fatty acids of sunflower-seed oil between monoene and polyene fatty acid fractions

In case of selectively hardened sunflower-seed oil, at the beginning of hardening the trans isomer content is distributed in a ratio of 1 : 1 between oleic and linoleic acid, while this ratio is 3 : 1 when maximum trans isomer content has been reached. In the hardening process the trans isomer content of oleic acid rises from 18.3% to 63.9%, and that of linoleic acid from 8.5% to 133% (a value higher than 100% is possible, since the figures are obtained with reference to methyl elaidate).

If from the analysis of samples taken every 30 minutes the decrease in linoleic acid is compared to the increase in trans isomer content of the oleic acid fraction, almost identical values are obtained.

Table 8

Changes in the quantity of monoene — diene fractions and in the trans content of the monoene fraction during the selective hydrogenation of sunflower-seed oil

Time, minute	Increase in the monoene fraction, %	Decrease in the diene fraction, %	Increase of trans isomer in the monoene fraction, %
0— 30	3.3	4.2	2.5
30— 60	2.9	3.6	4.3
60— 90	9.4	10.1	10.2
90—120	10.5	10.0	9.7
120—150	13.5	13.6	10.7
150—180	2.9	3.1	3.1

Note: The percentage quantities refer to sunflower-seed oil.

It seems that under the conditions of selective hardening the trans isomer content of the monoene fraction increases substantially, simultaneously with the conversion of linoleic acid into oleic acid and this in such a way that while one of the double bonds in linoleic acid becomes saturated the other is

isomerized. As the quantity of linoleic acid decreases, a higher proportion of unreacted trans isomeric fatty acids remains and the decrease in the conversion rate of linoleic → oleic acid during the hardening reaction can be explained by a higher quantity of trans isomers and by lower reactivity of the latter in the hydrogenation process.

*

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STUDY OF THE PROPAGATION OF BACTERIA ON LIQUID *N*-PARAFFIN AT ROOM TEMPERATURE

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The propagation of some non-identified soil bacteria on liquid *n*-paraffin at room temperature was studied. Propagation under the given conditions was most successful when strains No. 41 and No. 6061 were propagated on a nutrient medium containing 1% (w/v) paraffin and $(NH_4)_2HPO_4$ or $(NH_4)_2SO_4$ as the N source. With increasing paraffin concentration bacterial growth increased, however, the bio-mass yield related to paraffin decreased. The emulsifying agents applied: Genapol, Holstophan, Tween 80, Emulgin had no influence on the growth of bacteria. The use of yeast extract did not promote growth.

The paraffin samples containing $C_{14}-C_{17}$ and $C_{18}-C_{22}$ hydrocarbons, as well as the *n*-paraffin of *British Petroleum* (BP) were a better carbon source than a paraffin preparation containing $C_{10}-C_{13}$ hydrocarbons. No significant difference was found between growth when fractions $C_{14}-C_{17}$ and $C_{18}-C_{22}$ and BP *n*-paraffin were used in the medium. The specific growth rate for the CHEMAP fermentor was found to be 0.27 h^{-1}, the generation time 2.57 h.

The difference between fermentations carried out in the CHEMAP and KUTESZ fermentors was not significant.

In recent years great efforts were made the world over to find new sources of food and feed of high protein content. An important role is played in this respect by the bio-mass of various non-pathogenic microorganisms grown on media containing raw materials not utilized as food or feed. Thus high- intensity investigations were carried out to utilize hydrocarbons microbiologically, in particular in the production of bio-mass of high protein content.

Though because of technical difficulties arising in the propagation of bacteria the intensity of research is lower than that on yeasts, the bio-mass obtained with bacteria is of higher value, according to WAGNER (1970) than the result of yeast propagation on *n*-paraffin.

The review of related literature has shown that the different bacterial strains utilize hydrocarbons of different chain length. TRECCANI (1965) found the *Pseudomonas aeruginosa* bacterium to assimilate hydrocarbons of 6—8, 11—16 and 24—28 carbon number. A soil-bacterium was isolated by KRASILNIKOV & STEPANOVA (1971) which utilized hydrocarbons of C_{14}—C_{17} chain length. The bacterial strains isolated by OUYAMA & FOSTER (1965) utilized all hydrocarbons of C_1—C_{22}. YANAGAWA (1972) propagated a *Corinebacterium simplex* strain on C_{14}—C_{17} hydrocarbons. LUKINS and FOSTER (1963) found difference in the hydrocarbon assimilating capacity within the same bacterial

species. FUHS (1961) found that while some bacterial strains, whithin the species *Pseudomonas aeruginosa*, assimilated *n*-paraffins of various carbon chains, some other strains were capable of assimilating aromatic hydrocarbons. As seen from the work of SEELER (1962) and LUKINS and FOSTER (1963), hydrocarbons of medium carbon number are more difficult to assimilate than those of longer chains. FRIDERICKS (1966) studying several bacterial strains observed that on media containing *n*-paraffins of C_5—C_8 growth was poor. Various explanations were put forward by different authors for the low assimilation of medium-chain hydrocarbons. LUKINS and FOSTER (1963) assume that this phenomenon is related to the toxic effect of medium-length carbon chains, or to the fact that the bacteria cannot assimilate the metabolic products of these hydrocarbons. FUHS (1961) relates the phenomenon to the different spatial configuration of these hydrocarbons.

The aim of this study is the investigation of factors influencing propagation of bacteria on liquid *n*-paraffin media, containing hydrocarbon chains of varied length.

1. Materials and methods

1.1. Bacteria used

Non-identified bacteria of various origin were used in the experiments. The 6—8-hour-old culture of No.6061 strain consisted of a homogeneous mass of short rods of 1—2 μm. Strain No.41 consisted of flagellate rods of 1 $\times$3 μm (Fig. 1). Both strains are non-spore-forming.

1.2. n-paraffins used as C source

The composition of the *n*-paraffins used in the experiments, as determined by gas chromatography at the *Hungarian Research Institute for Mineral Oil and Natural Gases*, was as shown in the Table on p. 373.

1.3. Nutrient medium

The composition of the nutrient medium, in addition to 1 % (w/v) *n*-paraffin, was as follows:

KH_2PO_4	0.7 g $\cdot$ l^{-1}
$MgSO_4 \cdot 7H_2O$	0.8 g $\cdot$ l^{-1}
NaCl	0.5 g $\cdot$ l^{-1}

Various materials were used as N source, in every case at a concentration of 0.3 g N per litre medium. In experiments where the effect of paraffin con-

Percentage composition of preparations

Chain-length	BP	NP-4-70	NP-5-70	NP-6-70
Below C_9	—	0.1	—	—
C_9	—	0.4	—	—
C_{10}	—	16.2	—	—
C_{11}	—	47.5	—	—
C_{12}	—	28.4	—	—
Below C_{13}	0.4	—	—	—
C_{13}	3.8	5.0	1.6	—
C_{14}	30.1	0.5	22.0	—
C_{15}	28.3	—	37.3	—
C_{16}	22.6	—	24.8	0.2
C_{17}	14.2	—	9.3	3.8
C_{18}	0.7	—	2.2	27.9
C_{19}	—	—	—	33.2
C_{20}	—	—	—	23.5
C_{21}	—	—	—	8.4
C_{22}	—	—	—	1.0
Materials of non *n*-paraffin character	—	1.8	2.2	2.0
Pour-point, °C	+8	−25	+10	+30

BP signifies British Petroleum.
NP signifies Hungarian Research Institute for Mineral Oil and Natural Gases.

centration was investigated the N concentration was increased in proportion to the C source.

The compounds used as N source were the following: KNO_3, NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$, urea and $(NH_4)_2HPO_4$.

1.4. Emulsifying agents

The emulsifying agents used were as follows:

Genapol (*Anorgana*-Gendorf)
Holstophan (*Hoechst*)
Tween 80 (*Goldschmidt*)
Emulgin 0.5

1.5. Dry matter content of the cells

To determine the dry matter content of the cells a Sartorius membrane-filter of 0.4 mm mesh was used. Prior to the determination the filter was dried to constant weight in a drying oven of 60—70°C, weighed and placed in a Zsigmondy-filter. 10 ml of the fermentation liquor was thoroughly shaken and filtered. The bio-mass was first washed with 15 ml distilled water, then with 5 ml of 10% hydrochloric acid, to remove residual salts. Finally it was washed again with 15 ml distilled water. To remove the residual *n*-paraffin

the bio-mass was washed with petroleum ether and then dried to constant weight in a drying oven at 70°C.

To determine the cell concentration, upon completion of the fermentation the fermentation liquor was centrifuged in a laboratory centrifuge of 13 500 rpm for 15 minutes (Type FC 45) and in a supercentrifuge at 30 000 rpm (*Machine Factory for the Chemical Industries*, Budapest).

1.6. Determination of raw protein, residual hydrocarbons and lipoids

The raw protein content was determined from the bio-mass dried to constant weight by Kjeldahl's method. The method described by KERSTER (1967) was used to determine lipoids and residual hydrocarbons. 150 ml of a 1 : 1 mixture of chloroform and methanol was added to 1—2 g of dried bio-mass and the extraction was carried out twice. The extract was then washed with distilled water and evaporated to dryness. The residue was dissolved in ether, washed again with distilled water, evaporated and after adding carbon tetrachloride, applied to a silica-gel column. The lipoids are adsorbed on the column, while the hydrocarbons are removed with the solvent. After evaporating the solvent, the hydrocarbons were determined by weighing. The lipoid content is established by extracting the amount of hydrocarbons from the residue of ether extract after evaporation of the ether.

1.7. Preparation of the inoculum

The bacteria propagated on solid medium were suspended in a liquid synthetic medium containing 1% *n*-paraffin and, to adapt, cultured at 35°C for 48 hours under shaking. The cells obtained by centrifuging were used as the inoculum.

1.8. Methods of propagation

The temperature applied in shake-cultures as well as in the experiments carried out in the different fermentors, was 35°C. Temperatures differing from this are indicated.

1.8.1. Propagation in shake-culture. A horizontal type shaking apparatus was available. Erlenmeyer flasks of 250 ml capacity, each containing 40 ml nutrient medium, were used. The pH of the nutrient was controlled twice daily, and adjusted to pH 7 according to need with a 5% NH_4OH solution. The cultures were shaken at 180 rpm corresponding to an oxygen dissolution rate of 30 mmoles per litre per hour.

1.8.2. Propagation in a KUTESZ fermentor of 10 litre capacity. To obtain a larger quantity of bacteria 10-litre *KUTESZ* glass fermentors were applied. 3 000 ml nutrient was fermented in each. The temperature of the

fermentors, placed in a water bath, was automatically controlled and the amount of oxygen entering the fermentor was measured with a rotameter. 3 litres oxygen per minute were sparged into the liquor with an agitator of 1 000 rpm, corresponding to an oxygen dissolution rate of 140 mmoles per litre per hour. Samples for analysis were removed with a syringe through the rubber tube protruding into the fermentor. The pH was controlled hourly and adjusted with 5% ammonium hydroxide to pH 7, if necessary.

1.8.3. Propagation in a 20-litre CHEMAP fermentor. 4 litres of nutrient were introduced into the fermentor (Type F 0020). pH is controlled automatically in the fermentor by the addition of 10% ammonium hydroxide. The agitator speed was 1 000 rpm and the sparged air 4 litres per minute, corresponding to an oxygen dissolution rate of 165 mmoles per litre per hour. To ensure even sparging of the air, an inlay of several stages was inserted. To increase the dispersion of *n*-paraffin and air in the nutrient open, two-spout fittings were fixed to the shaft of the agitator, enclosed in a circulation tube. The circulating tube was perforated and the inflow spouts were between the agitators. The air and the *n*-paraffin formed a fine dispersion in the whole space of the apparatus.

1.8.4. Propagation in tube fermentor. The tube fermentors used were of 1 litre capacity and the air dispersed in them through a Jena Gl sintered glass filter. Temperature was controlled with the help of an ultrathermostat. Samples were taken through the rubber tube protruding into the fermentor, with a syringe. pH was controlled hourly and it was adjusted with a 5% NH_4OH solution. 400 ml of air per minute were introduced into the nutrient, measured by rotameter, corresponding to an oxygen dissolution rate of 70 mmoles per litre per hour.

2. Results

2.1. Bacterial growth as affected by the kind of N source applied

According to the literature various nitrogen sources may be applied in the propagation of hydrocarbon assimilating bacteria. GRECHUSHKINA and NIKITINA (1965) found sodium nitrate to be the best source from the point of view of growth. Besides $NaNO_3$, potassium nitrate (KOSHELEVA *et al.*, 1965; THIJSSE & VAN DER LINDER, 1958), ammonium sulphate (YAMANADA, 1963), ammonium nitrate (ZAHN, 1968; SHAH & BUTT, 1970) ammonium chloride (CHAMPAGNAT, 1968) were suggested. Thus we found it desirable to study the effect of various N sources on the growth of bacteria.

Strains No.41 and No.6061 were propagated in shake-culture on a nutrient containing 1% *n*-paraffin (BP) at 35°C and pH 7.

The growth of Strain No. 41 as a function of the N source is shown in Table 1.

Table 1

Effect of the N source on the cell concentration of shake-cultures on (BP) n-paraffin containing media (Strain No. 41)

(96-hour-fermentation at 35 °C, 1% *n*-paraffin, 0.3% N)

N source	$\bar{x}$	s
$(NH_4)_2HPO_4$	0.290	0.009
$(NH_4)_2SO_4$	0.288	0.008
NH_4Cl	0.217	0.006
NH_4NO_3	0.189	0.009
KNO_3	0.103	0.004
urea	0.091	0.008

$\bar{x}$ = mean value of cell concentration, % (w/v).
s = standard deviation.
n = 5.

Significance of differences between mean values obtained with different N sources

	$(NH_4)_2HPO_4$	$(NH_4)_2SO_4$	NH_4Cl	NH_4NO_3	KNO_3
$(NH_4)_2SO_4$	Ø				
NH_4Cl	xxx	xxx			
NH_4NO_3	xxx	xxx	xxx		
KNO_3	xxx	xxx	xxx	xxx	
urea	xxx	xxx	xxx	xxx	x

Ø = difference not significant.
x = difference significant ($\alpha \leq 0.05$).
xxx = difference very highly significant ($\alpha \leq 0.001$).

Analysis of variance has shown a significant difference between the growth of bacteria with various N sources.

Urea gave significantly lower results than KNO_3, NH_4Cl, NH_4NO_3, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$. $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ were significantly better than the other N sources. The difference between the results obtained with $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ was not significant, according to the *t* test.

The growth of Strain No. 6061 as a function of N source is summarized in Table 2.

Results of the analysis of variance are shown in the Table. The difference between N sources was found highly significant. KNO_3 gave significantly higher results than NH_4Cl or $(NH_4)_2SO_4$, while the difference was not significant in comparison to NH_4NO_3, according to the *t* test.

Table 2

Effect of the N source on the cell concentration of shake-cultures on (BP) n-paraffin containing media (Strain No. 6061)

(96-hour- fermentation at 35 °C, 1% *n*-paraffin, 0.3% N)

N source	$\bar{x}$	s
$(NH_4)_2HPO_4$	0.266	0.013
$(NH_4)_2SO_4$	0.259	0.008
KNO_3	0.247	0.009
NH_4NO_3	0.245	0.011
NH_4Cl	0.236	0.006
urea	0.192	0.013

$\bar{x}$ = mean value of cell concentration, % (w/v).
s = standard deviation.
n = 5.

Significance of differences between mean values obtained with different N sources

	$(NH_4)_2HPO_4$	$(NH_4)_2SO_4$	KNO_3	NH_4NO_3	NH_4Cl
$(NH_4)_2SO_4$	Ø				
KNO_3	x	x			
NH_4NO_3	x	x	Ø		
NH_4Cl	xxx	xxx	x	Ø	
urea	xxx	xxx	xxx	xxx	xxx

Ø = difference not significant.
x = difference significant ($\alpha \leq 0.05$).
xxx = difference very highly significant ($\alpha \leq 0.001$).

Of the N sources tested $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ proved to be significantly better, but between these two sources the difference was not significant.

As seen from the results the N source applied is important from the point of view of growth.

2.2. Influence of temperature on the growth of bacteria

The optimum growth temperature of hydrocarbon assimilating bacteria was found to be 25—40°C. KVASNIKOV (1964) succeeded in finding a thermophilic aerobic spore forming bacterial strain with an optimum growth temperature of 45—65°C. The optimum growth temperature of the thermophilic bacteria isolated by SLAVNINA (1963) was 40—55°C.

In preliminary experiments bacteria from the strain collection of the Institute were grown on solid nutrient medium containing *n*-paraffin, at various

temperatures. The mean values characterizing the size of bacterial colonies, obtained at 20°C, were very low. At 50°C none of the strains formed colonies. Thus strains Nos. 41 and 6061 were tested again by propagating in shake-culture, using the most advantageous N source. pH was adjusted by the addition of ammonium hydroxide, if needed. The temperature was set between 25 and 45°C. Results are shown in Fig. 2.

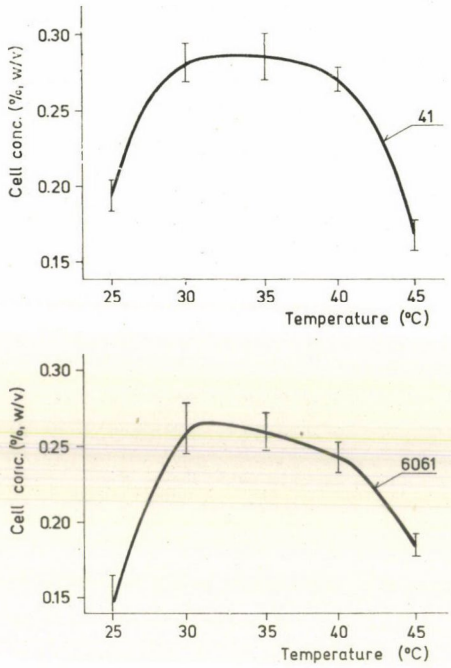

Fig. 2. Effect of fermentation temperature on the growth of bacterial Strains No. 41 and No. 6061 in shake culture. Fermentation period: 96 hours; N source: $(NH_4)_2HPO_4$, pH = 7, in a medium containing 1% (w/v) BP paraffin. I = 95% confidence limits

When plotting the results, the 95% confidence limit was also shown. On the basis of mathematical statistical analysis of the results it was established that for the fermentation of Strains Nos. 41 and 6061 the temperature range between 25 and 45°C was significantly worse than fermentation at 30°C. On the other hand in the 30—40°C range there was no significant difference between fermentations at the various temperatures. Thus it was decided to perform the fermentation experiments with both strains at 30°C.

2.3. The growth of bacteria as affected by the pH of the nutrient medium

Generally the nutrient medium containing *n*-paraffin is set at a neutral pH. According to SLAVNINA (1963) the optimum pH for thermophilic bacteria is 6.2.

The effect of pH on the growth of bacteria was tested in the range of pH 5—9 at intervals of 0.5. Growth was established after a 48-hour fermentation period. The best N source and *n*-paraffin as carbon source were used. Results are shown in Fig. 3.

On plotting the results the 95% confidence limits were shown for every mean value. As seen from the results for Strain No. 41 optimum pH value was 6.0—7.5, for Strain No. 6061 between pH 6.0 and 7.0. Thus in the experiments both strains were propagated at pH 7.0.

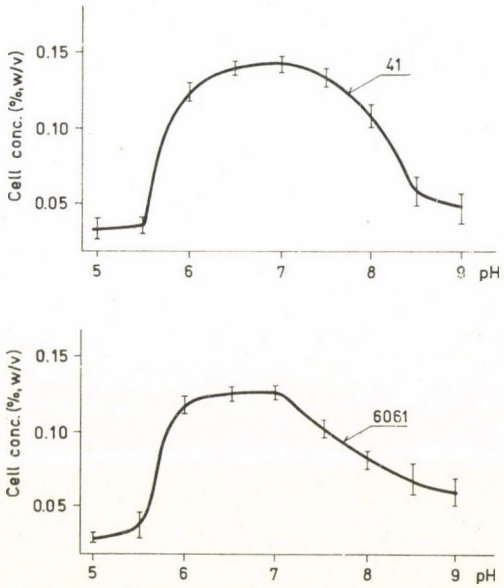

Fig. 3. Effect of pH on growth of Strains No. 41 and No. 6061, in shake-culture. Fermentation period: 48 hours; temperature: 35° C; N source: $(NH_4)_2HPO_4$ in a medium containing 1% (w/v) BP paraffin. I = 95% confidence limits

2.4. The effect of n-paraffin concentration

Takeo and his co-workers (1969) have shown that the amount of *n*-paraffin, assimilated per unit fermentation liquor, depends on the way of addition. The amount of hydrocarbons added may be varied between a few tenth and 10%.

The effect of the *n*-paraffin concentration in the fermentation liquor on growth and yield of bacterial Strains No. 41 and No. 6061 was studied at optimum pH, temperature and N source. The applied concentrations were 1, 2 and 3% (w/v). The analysis of the results was performed according to the method of cultivation applied (shake-culture, *KUTESZ* fermentor).

Results are shown in Tables 3 to 5.

Table 3

Effect of paraffin concentration in the fermentation liquor on growth and yield of bacteria in shake-cultures (Strain No. 41)

(Fermentation at 35 °C, pH = 7; N source: $(NH_4)_2HPO_4$)

Paraffin concentration (BP), %	Cell concentration, % (w/v)	Yield, %		Fermentation period, hour
		$\bar{y}$	s	
1	0.283	40.66	1.77	96
2	0.428	30.51	1.10	140
3	0.655	31.16	0.61	195

n = 8.
$\bar{y}$ = mean yield.
s = standard deviation.

Table 4

Effect of paraffin concentration in the broth on the growth and yield of bacteria in shake-culture (Strain No. 6061)

(Fermentation performed at 35 °C, pH = 7; N source: $(NH_4)_2HPO_4$)

Paraffin concentration, %	Cell concentration % (w/v)	Yield, %		Fermentation period, hour
		$\bar{y}$	s	
1	0.266	37.85	2.04	96
2	0.404	28.89	1.57	140
3	0.615	28.98	0.87	195

n = 8.
$\bar{y}$ = mean of yields.
s = standard deviation.

Table 5

Effect of paraffin concentration in the broth on growth and yield of bacteria in the KUTESZ fermentor (Strain No. 41)

(Fermentation performed at 35 °C, pH = 7; N source: $(NH_4)_2HPO_4$)

Paraffin concentration, %	Cell concentration % (w/v)	Yield, %		Fermentation period, hour
		$\bar{y}$	s	
1	0.4825	68.98	4.11	48
2	0.5675	40.75	1.72	80
3	0.6325	30.08	2.26	100

n = 4.
$\bar{y}$ = mean value of yields.
s = standard deviation.

As seen in Tables 3 and 4 the propagation of both strains in shake-culture resulted in increasing yield with the increase of the *n*-paraffin concentration, however, the yields related to the paraffin content decreased. The same observation was made when cultivation was performed in the fermentor (Table 5). Under the experimental conditions applied 1% *n*-paraffin proved to be the most suitable concentration, since at this level a good yield was achieved. It was observed during the experiments, that with both methods of propagation increasing *n*-paraffin concentration entailed increased fermentation time requirement.

2.5. Growth as affected by the addition of emulsifying agents

It was assumed that by increasing the degree of dispersion of the *n*-alkanes the growth rate may be increased, therefore the effect of various emulsifying agents was tested with Strain No. 41.

The emulsifying agents were assumed to increase the degree of dispersion of paraffins while not inhibiting the growth of bacteria. The experiments were performed in shake-cultures at 35°C, pH 7 and $(NH_4)_2HPO_4$ used as nitrogen source. The emulsifying agents tested were: Genapol (*Anorgana*-Gendorf), Holstophan (*Hoechst*), Tween 80 (*Goldschmidt*), Emulgin 0.5 at a concentration of 70 ml $\cdot$ l^{-1}. In every series of experiments a fermentation without emulsifying agent was included for control. Microscopic tests during fermentation have shown the paraffin drops to be smaller in nutrients with added emulsificator.

The results of these experiments are given in Table 6.

Table 6

Effect of emulsifying agents on growth of Strain No. 41
(Fermented at 35 °C, pH = 7; N source: $(NH_4)_2HPO_4$; fermentation period: 96 hours)

Carbon source	Control		Emulsifying agents							
			Tween 80		Emulgin 0.5		Genapol		Holstophan	
	$\bar{x}$	s	$\bar{y}$	s	$\bar{y}$	s	$\bar{y}$	s	$\bar{y}$	s
NP-4-70										
C_0-C_{13}	0.148	0.015	0.143	0.011	0.140	0.014	0.143	0.015	0.148	0.013
NP-5-70										
C_{14}-C_{17}	0.264	0.031	0.262	0.013	0.254	0.023	0.256	0.031	0.264	0.016
NP-6-70										
C_{18}-C_{22}	0.300	0.012	0.292	0.009	0.280	0.021	0.276	0.032	0.284	0.020
BP										
C_{13}-C_{18}	0.297	0.007	0.291	0.012	0.281	0.011	0.291	0.007	0.293	0.008

$\bar{x}$, $\bar{y}$ = mean value of cell concentration % (w/v).
s = standard deviation of the mean.
n = 8.

Comparison of the results shows that the difference between bacterium yields obtained with the various emulsifying agents and the control is not significant. This observation was supported by the fact that the decrease in pH due to oxidation of alkanes to acids occurred every time after a period of the same length. The same period was required also for the acids to become assimilated, entailing the stabilization or even increase of the pH value.

It was concluded from the experiments that the emulsifying agents used at the given concentration did not inhibit bacterial growth but did not increase the efficiency of fermentation either, therefore their application was considered unnecessary.

2.6. Influence of yeast extract on bacterial growth

Some authors have added yeast extract or vitamins to the medium used for propagation (CHAMPAGNAT, 1968).

To investigate this problem we used shake-cultures of Strain No. 41 at 35°C. The medium was prepared with carbon chains of different length. The yeast extract was added to the medium at a concentration of 0.01 %. A fermentation performed in the same way, but without yeast extract served as the control.

Table 7 shows the effect of yeast extract on growth.

According to the *t* test significant difference was not found between the control or anyone of the fermentations. Thus yeast extract was not used in further experiments.

Table 7

Effect of yeast extract on growth of Strain No. 41

(Fermentation temperature 35 °C, pH = 7; N source: $(NH_4)_2HPO_4$; fermentation period: 96 hours)

Carbon sources	Yeast extract		Control	
	$\bar{x}$	s	$\bar{y}$	s
NP-6-70	0.296	0.011	0.298	0.009
BP	0.292	0.008	0.294	0.011
NP-5-70	0.272	0.014	0.269	0.013

$\bar{x}$, $\bar{y}$ = mean value of cell concentration, % (w/v).
s = standard deviation of the means.
n = 8.

2.7. Comparison of the amount and quality of yield obtained on media containing different n-paraffins

The *n*-paraffins obtained partly from the *Hungarian Research Institute for Mineral Oil and Natural Gases* and from *British Petroleum* were compared. The composition of the *n*-paraffins is given in para.1.2.

Strain No. 41 was used in the experiments. When fermentation was finished, the protein, lipoid and residual hydrocarbon contents of the bio-mass were determined.

Many authors have reported on the composition of bacteria grown on *n*-paraffins. According to HUMPHREY (1970) bacteria grown on *n*-paraffin had non-extracted protein content of 62—73% and a lipoid content of 10—15%. YANAGAWA (1972) found 13% lipoid in the bio-mass of bacteria grown on C_{14}—C_{16} hydrocarbons.

The paraffin concentration applied in the experiments was 1% (w/v) and $(NH_4)_2HPO_4$ was used as the N source. Propagation was carried out in shake-cultures and agitated fermentors. The yields related to the various paraffins are shown in Tables 8 and 9.

Table 8

Comparison of growth on media containing different paraffin samples, using Strain No. 41 in shake-culture

(Fermentation temperature 35 °C, pH = 7; N source: $(NH_4)_2HPO_4$; fermentation time: 96 hours)

Carbon source	$\bar{x}$	s
NP-6-70	0.289	0.014
BP	0.284	0.015
NP-5-70	0.271	0.016
NP-4-70	0.167	0.009

$\bar{x}$ = mean cell concentration, % (w/v).
s = standard deviation of the mean value.
n = 5.

Significance of difference between cell concentrations obtained on different paraffin samples

	NP-6-70	BP	NP-5-70
BP	Ø		
NP-5-70	Ø	Ø	
NP-4-70	xxx	xxx	xxx

Ø = difference not significant.
xxx = difference very highly significant ($\alpha \leq 0.001$).

Table 9

Comparison of growth on media containing different paraffin samples, using Strain No. 41 in the KUTESZ fermentor

(Fermentation temperature 35 °C, pH = 7; N source: $(NH_4)_2HPO_4$)

Carbon source	$\bar{x}$	s
BP	4.732	0.497
NP-6-70	4.550	0.404
NP-5-70	4.325	0.639
NP-4-70	1.425	0.427

$\bar{x}$ = mean value of cell concentration, $g \cdot l^{-1}$.
s = standard deviation.
n = 4.

Significance of differences between mean values of cell concentrations obtained on different paraffin samples

	BP	NP-6-70	NP-5-70
NP-6-70	Ø		
NP-5-70	Ø	Ø	
NP-4-70	xxx	xxx	xxx

Ø = difference not significant.
xxx = difference very highly significant ($\alpha \leq 0.001$).

Analysis of variance of the results has shown that the yield obtained with both methods of fermentation on C_{10}—C_{13} containing media was significantly lower than those obtained on C_{14}—C_{17}, C_{18}—C_{22} or BP *n*-paraffin containing media. There was no significant difference between results obtained on C_{14}—C_{16} and C_{18}—C_{22} or on BP paraffin.

The result of analysis of the bio-masses is summarized in Tables 10 and 11.

Table 10

Raw protein and residual hydrocarbon content of bacteria propagated on media containing different paraffins (Strain No. 41)

(Fermentation temperature 35 °C, pH = 7; N source: $(NH_4)_2HPO_4$)

Carbon source	Raw protein, %	s	Residual hydrocarbon	
			%	s
BP	60.25	1.55	0.200	0.022
Np-4-70	60.12	0.85	0.234	0.026
NP-5-70	60.10	1.43	0.215	0.031
NP-6-70	60.10	0.67	0.202	0.022

s = standard deviation.

Table 11

Lipoid content of bacteria grown on media containing different paraffins (Strain No. 41)

(Fermentation temperature in the KUTESZ fermentor: 35 °C, pH = 7, fermentation time: 48 hours)

Carbon source	Lipoid content, %	s
NP-6-70	11.76	0.960
BP	10.13	0.760
NP-4-70	9.67	0.650
NP-5-70	9.40	0.173

s = standard deviation.

The average raw protein content of the bio-masses was 10% with no significant difference between individual fermentations.

There was no significant difference in the residual hydrocarbon content of the samples either. The residual hydrocarbon content related to the solids content of the cells amounted to about 0.2—0.3%.

The average amount of lipoids was 9.40—11.76%, however the bio-mass grown on C_{18}—C_{22} was significantly richer in lipoids than the rest.

It was concluded from the experiments that C_{10}—C_{13} paraffins are not suitable for the propagation of Strain No. 41. Probably the low carbon number paraffins, being volatile, are carried away by aeration.

2.8. Comparison of the fermentors on the basis of the obtainable yield

Using the fermentors as described in para.1.7.1. fermentation experiments were carried out on 1% *n*-paraffin (BP) containing medium, at optimum temperature. The yields obtained in 48-hour fermentations were compared and tabulated in Table 12.

Table 12

Comparison of different fermentation techniques on the basis of bacterium concentration

(Fermentation temperature: 35 °C, pH = 7, fermentation time: 48 hours)

	Shake-culture	Tube	KUTESZ	CHEMAP
		fermentor		
$\bar{x}$	1.403	2.93	4.76	4.10
s	0.027	0.15	0.32	0.34

n = 3.
$\bar{x}$ = mean value of cell concentrations, $g \cdot l^{-1}$.
s = standard deviation.

Significance of differences between cell concentrations obtained in the different fermentors

	Tube	KUTESZ	CHEMAP
	fermentor		
Shake-culture	xxx	xxx	xxx
Chemap fermentor	xxx	Ø	
Kutesz fermentor	xxx		

Ø = difference not significant.
xxx = difference very highly significant ($\alpha \leq 0.001$).

The mathematical statistical analysis of the results has shown significant differences between the individual fermentation techniques. Shake-cultures gave significantly lower results than the tube fermentor, the 10- or 20-litre agitated fermentors, respectively. This was due probably to the conditions of aeration being more advantageous in the latter fermentors. No significant difference was observed between growth in the *KUTESZ* fermentor and the *CHEMAP* fermentor.

The yield obtained in the tube fermentor was significantly lower than in the *KUTESZ* and *CHEMAP* fermentors, respectively. It was established that the useful capacity of the latter two fermentors cannot be increased, because of the intense foaming during fermentation. Foaming in the tube fermentor was also extremely extensive and it could not be inhibited by the addition of antifoaming agents, thus the use of this type of fermentor was omitted in further experiments.

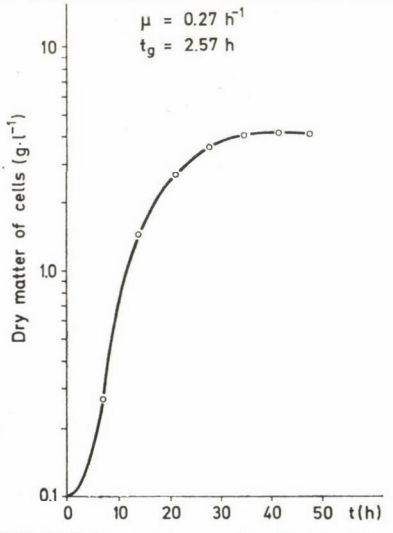

Fig. 4. Growth curve in CHEMAP fermentor, using Strain No. 41. N source: $(NH_4)HPO_4$, C source: 1% (w/v) BP paraffin

The growth curve of Strain No. 41 in the *CHEMAP* fermentor was plotted on a semi-logarithmic scale (Fig. 4).

As seen in the figure the exponential phase of growth was reached in about 5 hours. The specific growth rate in the exponential phase (μ) and generation time (t) were calculated:

$$\mu = 0.27 \ h^{-1} \qquad t = 2.57 \ h$$

3. Conclusions

The experiments have shown the source of nitrogen to be an important factor of growth. Strains No. 41 and No. 6061 were propagated with the best result in fermentation liquors containing $(NH_4)_2HPO_4$ or $(NH_4)_2SO_4$ in addition to 1% *n*-paraffin. The optimum fermentation temperature was found to be between 30 and 40°C. The experimental fermentations were performed at 35°C.

For Strain No. 41 the optimum pH range was 6.0—7.5, while for No. 6061 it was 6.0—7.0.

It was established that with the increase of paraffin concentration the bacterium yield increases in absolute value, however, related to the paraffin concentration in the medium it decreases. In the available fermentors, of the 1, 2 and 3% paraffin concentrations applied, 1% proved to be the most suitable.

The emulsifying agents applied did not inhibit growth, but did not improve it either.

The addition of yeast extract did not improve yields as compared to that of the control fermentation.

Of the paraffin samples used the one containing hydrocarbon chains of C_{10}—C_{13} gave lower result than either the Hungarian samples of C_{14}—C_{17} and C_{18}—C_{22} or the *British Petroleum* *n*-paraffin. There was no significant difference between the latter three samples.

The analysis of the bio-masses grown on media containing different paraffin samples did not reveal significant differences in the raw protein or the residual hydrocarbon content. As regards lipoids, the bio-mass obtained on C_{18}—C_{22} differed significantly from the rest.

The comparison of the various paraffin samples has shown C_{10}—C_{13} hydrocarbons to be unsuitable for the propagation of bacterial Strain No. 41.

When comparing the various fermentation techniques fermentation in shake-cultures proved to be significantly lower than in tube, *KUTESZ* or *CHEMAP* fermentors, respectively. The yield of tube fermentation was significantly lower than that of the *KUTESZ* or *CHEMAP* fermentors. The difference between the yields obtained in the *KUTESZ* and the *CHEMAP* fermentors was not significant. The specific growth rate in the *CHEMAP* fermentor was 0.27 h^{-1}, while the generation time had a value of 2.57 h.

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Fig. 1. Electron-microscopic picture of bacterium Strain No. 6061

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DETERMINATION OF THE BETA-CAROTENE CONTENT OF THE GRAIN OF YELLOW ENDOSPERM-TYPE SORGHUMS

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The modified (HORVÁTH *et al.*, 1971) HAGER and MEYER-BERTENRATH (1966) thin-layer chromatographic method has been made applicable to the determination of the β-carotene content of sorghum grains containing minimum quantities of vitamin A precursors.

The pigment components were extracted with a 3 : 1 mixture of acetone : ether and saponified with KOH. The alkali was removed by washing with distilled water and the ether extracts dried over NaCl. The pigments were run on layers prepared according to HAGER and MEYER-BERTENRATH (1966) from a 50 : 50 : 40 mixture of petroleum ether : acetone : chloroform. After drying the layers, the spots of β-carotene were scraped off the plate, dissolved in chloroform and measured spectrophotometrically. Identifications were performed with the aid of a calibration curve.

This method made it possible to determine the β-carotene content of the available sorghums.

The following facts were established:

1. The yellow colour does not necessarily indicate high β-carotene content.
2. Many lines of the yellow endosperm varieties, at first thought to be homogeneous, later proved to be highly heterogeneous.
3. Some homogeneous lines of high β-carotene content were found which serve as the starting material for quality improvement.

The vitamin A precursors of the vegetable kingdom are 40 carbon-atom isoprenoid compounds. All carotenoids containing a non-substituted β-ionone ring residue or in which the β-ionone ring in position 5,6 is epoxidized possess vitamin A activity (Table 1; GOODWIN, 1955).

By way of oxidative cleavage provitamins are converted into vitamin A. The most efficient precursor is β-carotene containing two β-ionone rings from which two vitamin A molecules are formed. The other precursors are only half as efficient as β-carotene, since they contain only a single β-ionone ring.

The leaves of higher plants always contain vitamin A precursors, while of the grain fodders only maize (SPECTOR, 1956) and the yellow endosperm-type grain sorghums have a certain vitamin A precursor content. Maize contains α-, β- and γ-carotene and cryptoxanthine, but sorghum contains only β-carotene in a measurable quantity.

Animals satisfy their needs of vitamin A from vegetable sources in the form of precursors. Most animals convert these precursors already with the help of their intestinal villi into vitamin A, while in a smaller group of animals

this conversion takes place only in the liver. In the latter the bulk of vitamin A is stored in the *Kuppfer* cells and in the mitochondria. In the summer the animals receive sufficient amounts of green fodder, so that in this season there is usually no vitamin A deficiency, but in the winter, when vitamin A sources

Table 1

Carotenoids with vitamin A activity in the vegetable kingdom

Lower plants	Higher plants
Aphanicine	Beta-carotene and its 5,6-epoxide
Aphanine = myoxanthine	Alpha-carotene and its 5,6-epoxide
Torularhodine	Gamma-carotene
	Mutatochrome
	Cryptoxanthine
	Echinenone

are limited, there is a great danger of avitaminosis. This manifests itself in night-blindness, keratinization of the skin, general debility, reduced resistance to illness, and sometimes in mass death. This might be avoided partly by the proper siloing of green plants and by raising the carotenoid content of the grain fodders, and, on the other hand, by ensuring an extra vitamin A supply (*e.g.* from fodder carrot, fodder marrow, fodder cabbage or from synthetic preparations, *e.g.* from Aquital).

The objective of our work was to raise the β-carotene content of grain fodders, particularly of grain sorghum.

Commonly grown sorghums contain very little (only trace amounts) of β-carotene, while crossed with African yellow endosperm-type sorghums the β-carotene content can be considerably improved (from 0.3 to 1.5 ppm, Blessin *et al.*, 1958).

The first step was to become acquainted with the sorghum grain samples in our collection of varieties, particularly with the yellow endosperm-type sorghums.

Since the samples used as controls had a very low β-carotene content and quite often only very small quantities (10 to 20 g), were available from yellow endosperm-type inbred lines (totality of individuals within the same variety which have become homogeneous by self-pollination) intended for improve-

ment, it became necessary to develop a precise and sensitive method. For such very small quantities column chromatography is no longer sufficiently sensitive, so that we decided to use thin-layer chromatography.

1. Materials and methods

1.1. Materials

The β-carotene used for comparison was obtained from the firm *Hoffmann—La Roche.*

The other reagents were those marketed by *Reanal* (Hungary) as analytical grade reagents.

The investigated sorghum grain samples came from the collection of varieties of nursery sorghums which have been self-pollinated for several years in the Öthalom experimental farm of the Cereal Research Institute, Szeged.

1.2. Method

The thin-layer chromatographic method of HAGER and MEYER-BERTENRATH (1966) as modified by HORVÁTH and co-workers (1971) was adapted to suit the samples under investigation. The β-carotene of the investigated sorghum grains was identified by comparison with synthetic β-carotene on the basis of their R_f value, their colour, the shape of their spectra (HAGEN & MEYER-BERTENRATH, 1967a) and location of their absorption maxima (HAGEN & MEYER-BERTENRATH, 1967b).

Depending on the expected carotene content samples of 5 to 10 g of air-dry ground grain were used. The pigments were extracted with a 3 : 1 mixture of acetone : ether. From this mixture acetone was removed by washing with water and the ether extract saponified with KOH in methanol to a final concentration of 5% of alkali (HERTZBERG & JENSEN, 1966). The samples were allowed to stand overnight at temperatures varying between 0 and +5°C. Thus, after washing with distilled water the ether phase contained only the non-saponifiable lipoids (carotenoids, steroids), while, on the other hand alkaline treatment liberated also the esterified hydroxyl groups of the xanthophylls and could be easily identified by means of chromatography. The extract was dried over NaCl and evaporated in a rotational evaporator (*Rotadest* of MTA KUTESZ). The dry residue was taken up in ether and run on thin-layer plates prepared from a mixture of 29.5 g of calcium carbonate +6 g of magnesium oxide +5 g of calcium hydroxide and preliminarily activated at 110°C for one hour. The solvent was a 50 : 50 : 40 mixture of acetone : petroleum ether: chloroform. The spot of β-carotene was scraped off, eluted in

chloroform and after making the solution up to the desired volume photometrically determined at 464 nm in *Spectromom 360*. The results were evaluated with the aid of the calibration curve of synthetic β-carotene (Fig. 1) and calculated by accounting for the value of the loss factor of the thin-layer chromatographic method (0.395).

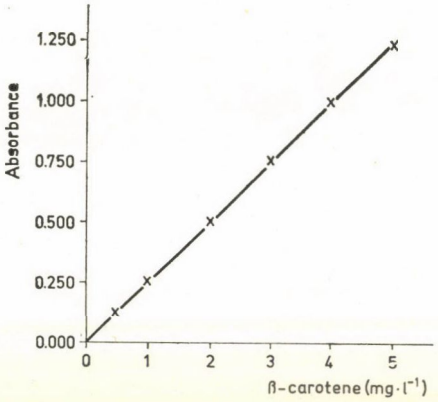

Fig. 1. Calibration curve of synthetic β-carotene dissolved in chloroform. Measured at 464 nm with "Spectromom 360"

2. Results

2.1. Procedure

By starting from a larger quantity (from 5 to 10 g instead of 2 g) of ground grain and by removing, after treatment with potassium hydroxide, the saponifiable lipoids, the modified method made it possible to determine the β-carotene content of extracts containing a lesser amount of impurities.

By means of these modifications we succeeded in determining the carotene content of sorghum grains with a minimum β-carotene content.

β-carotene was identified on the basis of its R_f value, its colour in visible light, the shape of its spectrum and the location of the absorption maxima.

The R_f value of the β-carotene obtained from sorghum grains was 0.84. The β-carotene had an orange colour in visible light and the absorption maxima in chloroform were found at 438, 464 and 489 nm. The shape of its spectrum facilitated its identification as crystalline β-carotene (Figs. 2 & 3).

2.2. Test results

Table 2 shows a characteristic group of the investigated sorghum grains. The data represent the results of two parallel measurements. The β-carotene content of the commonly grown sorghum samples was extremely low, but certain lines with high β-carotene content were also found (60—90—89—64,

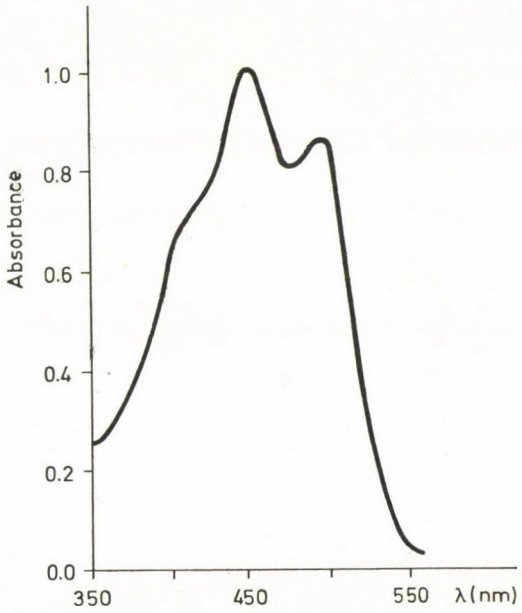

Fig. 2. Absorption curve of β-carotene dissolved in hexane

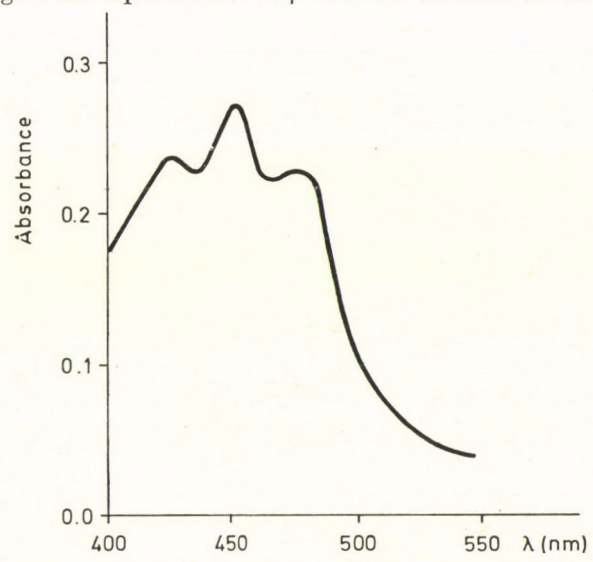

Fig. 3. Absorption curve of β-carotene isolated from the yellow (line 60 — 90 — 89 — 64) endospermium-type sorghum grain and dissolved in hexane. The pigments were isolated by thin-layer chromatography on Kieselgel-G plates. β-carotene content: 1.18 mg kg^{-1}

SGa-4, SGa-9). Among the supposedly homogeneous lines certain highly heterogeneous ones were discovered, *e. g.* K-453.

Table 3 shows the vitamin A requirements of various animals in IU and ppm of β-carotene. When converting IU into ppm β-carotene (1 IU = 0.3 μg

Table 2

Beta-carotene content of the investigated grain sorghum varieties
(averages of two parallels)

No.	Sorghum sample	β-carotene content in ppm	
		Mean value	Standard deviation
1.	60-90-89-64/72	1.18	0.042
2.	K-453/71 b	0.37	0.015
3.	S-Ga-9/71	0.36	0.014
4.	S-Ga-9/70	0.36	0.013
5.	S-Ga-4/71	0.33	0.013
6.	S-Ga-4/70	0.32	0.013
7.	Nadjada Aimg./71	0.17	0.007
8.	K-453/70 b	0.17	0.007
9.	C/70	0.11	0.004
10.	NK 222/71	0.10	0.004
11.	NK 222/70	0.09	0.004
12.	KG-63/71	0.09	0.004
13.	KG-63/70	0.09	0.004
14.	K-453/71 a	trace	
15.	K-453/70 a	trace	
16.	AK-9-2-65/69	trace	
17.	Hybar 242	trace	
18.	Hybar 456	trace	

Trace = less than 0.084 ppm.

Table 3

Vitamin A requirement of various animals

Animal	Feed	
	Vitamin A content ($IU \cdot kg^{-1}$)*	β-carotene content (ppm)
Pig (young)	6 000	1.80
Chicken	6 600	1.98
Hen	8 800	2.64
Cattle (young)	9 000	2.70
Cow (with calf)	13 500	4.05
Sow (in farrow)	30 000	9.00

* 1 IU (international unit) = 0.3 μg of β-carotene.

of vitamin A) we have taken into consideration the results of the feeding experiments of BURNS and co-workers (1951) who found that β-carotene is almost quantitatively converted into vitamin A.

Comparison of Tables 2 and 3 discloses the fact that the yellow endosperm-type sorghum grains with high β-carotene content are valuable sources for meeting the vitamin A requirement of animals.

3. Conclusions

By modifying the above mentioned method we succeeded in determining of the vitamin A activity of sorghum grains with minimum β-carotene content.

Table 2 shows that the yellow colour does not necessarily correspond to a high β-carotene content, moreover we have found a heterogeneous yellow endosperm-type line (K-453) within which, despite the yellow colour of the grain, the minimum β-carotene content is similar to that of commonly grown sorghum.

Hence, it is impossible to select sorghums of a high β-carotene content simply on the basis of their colour and without a suitable test method.

Table 2 shows further that there are certain yellow endosperm-type lines with high β-carotene content, which contain about 30 to 50 times more vitamin A precursor than the commonly grown sorghum with its minimum β-carotene content. Owing to this property these strains may serve as starting material for crops with improved quality.

*

The authors wish to thank the firm *Hoffmann–La Roche* for the β-carotene they kindly put at their disposal.

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CHANGES IN THE COLOUR AND ANTHOCYANIN CONTENT OF RASPBERRY FRUIT DURING RIPENING

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The anthocyanin content and colour of the fruits of three raspberry varieties were studied in various stages of ripening. It was found that in the course of ripening the spectrophotometrically measured anthocyanin content of fruits of the different varieties did not increase in an identical manner.

In the fruits of the *Knevett* variety the anthocyanin level increased uniformly as ripening progressed. Anthocyanin accumulation was more intensive in the first half of the ripening period of the *Findus 27* raspberry variety, while in the fruits of the *Bolgarski Rubin* variety far more anthocyanin accumulated in the second half of the ripening period than in other varieties. The over-ripe fruits of the last variety are characterized by a turn of the normal red-purple colour into dark violet. The absorption spectra of extracts prepared from fruits at different stages of ripeness showed no change, neither was a difference found in the number and ratio of anthocyanins separated by thin-layer or paper chromatography. It seems therefore probable that the undesirable deep purple colour of the over-ripe fruits of certain raspberry varieties is linked with an excessive anthocyanin accumulation. Thus, colorimetric tests and determination of the anthocyanin content during ripening of raspberries might offer a possibility of selecting varieties with more uniformly coloured fruits.

The characteristic colour of raspberry fruits is due to anthocyanin compounds. Though most fruits and other plant parts synthesize anthocyanins dependent on ecological and physiological factors (BLANK, 1958), certain quantitative differences within a species are more or less characteristic of the different varieties. BLASSE and FREYTAG (1958) investigated 34 red raspberry varieties by determining the extinction modulus which is proportional to the concentration of anthocyanins. They found significant differences between varieties. According to our own observations, however, there are sometimes greater differences between the colours of raspberry fruits of the same variety in subsequent stages of ripening than between the fruits of different varieties. A colour depending as little as possible on the stage of ripening is an important parameter both of freshly consumed and of preserved raspberries and also one of the aims of improvement. All this necessitated the study of the causes of colour changes taking place during raspberry ripening. Since, besides the quantity of anthocyanins, colour might depend also on the anthocyanin composition, pH, the presence of metal ions, flavonoids and colloid substances (BLANK, 1958), changes in some of these factors were studied in half-ripe, ripe and over-ripe fruits of certain raspberry varieties. The results of these investigations will be reported below.

1. Materials and methods

1.1. Fruits

The experiments were carried out with fruits of the *Bolgarski Rubin*, *Findus 27* and *Knevett* raspberry varieties at the Fertőd Research Station of the Horticultural Research Institute (Budapest). Fruits were collected at different stages of ripening. Fruits beginning to colour, but, without the firmness characteristic of fully ripened fruits, were considered as half-ripe. Fully developed fruits being ready for consumption were considered as ripe, while those of a deteriorated quality both in colour and firmness were classified as over-ripe. The selected stages represent a three- to four-day time interval.

1.2. Determination of pH

The juice was pressed from the fruits, filtered and diluted 1 : 1 with distilled water of pH 7. The pH of this solution was determined with a METROHM pH-meter using a combined electrode.

1.3. Extraction and investigation of anthocyanins

1.3.1. Extraction. From average samples of the fruits in different stages of ripening, extracts were prepared in a blendor with 0.1 *N* hydrochloric acid (10 ml hydrochloric acid per gram fresh weight). The extracts were filtered through a G3 glass filter. For chromatographic examination, the extracts were prepared with methanol containing 1 per cent of hydrochloric acid and after filtration, the extract was concentrated in a nitrogen stream and filtered again if necessary.

1.3.2. Colorimetry. The colour coordinates of the filtered extracts were determined with the MOMCOLOR colorimeter using a white standard and a cell correction.

1.3.3. Absorption spectrum, quantitative determination. The absorption spectra of the filtered extracts were recorded at every 5 nm with a UNICAM SP 500 spectrophotometer in the 400 to 600 nm range. Relative anthocyanin content was characterized by the optical density measured at the absorption maximum. In the 0.1 *N* hydrochloric acid extracts, λ_{max} was found to be at 525 nm, and in the extracts diluted with distilled water at 510 nm. The first value is in good agreement with that obtained by BLASSE and FREYTAG (1958), the second with that found by LAMORT (1958) and TANCHEV (1971).

1.3.4. Chromatography, densitometry. Depending on the anthocyanin content, 10 to 50 μl of the concentrated extract were applied either to Macherey-Nagel MN 300 cellulose layers (250 μ) or to MN 214 chromatographic paper.

The chromatograms were developed with 5 per cent formic acid at room temperature in the dark. The ratio of the anthocyanins was determined by densitometry of the spots separated on paper chromatograms with a ZEISS ERI-10 integrating densitometer. An absolute chemical identification of the individual anthocyanin components was not attempted, since the raspberry anthocyanins are chemically well defined (NYBOM, 1960; HARBORNE & HALL, 1964; GOMBKÖTŐ & PAIS, 1969; FRANCIS, 1972). But, for sake of certainty, the components eluted from the paper were roughly identified by means of partial hydrolysis (ABE & HAYASHI, 1956). For the identification of aglycones, pure cyanidin (product of FLUKA) was used.

2. Results

2.1. Colour changes during ripening

Figs. 1 to 3 show the colour coordinate changes of fruits of the *Bolgarski Rubin*, *Findus 27* and *Knevett* varieties at different stages of ripening.

The colour coordinates of the three varieties change in a characteristic manner. Fruits of the *Bolgarski Rubin* variety display the most conspicuous

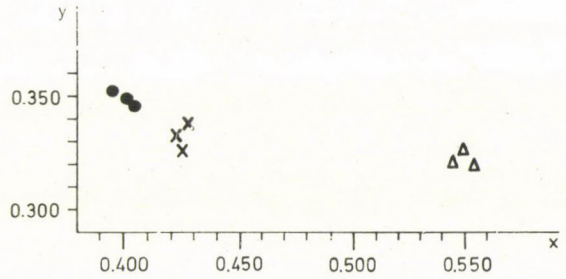

Fig. 1. Changes in the colour of *Bolgarski Rubin* raspberry fruits in the course of ripening. 0.1 *N* hydrochloric acid extract (1 g fresh weight per 10 ml). Three replicates measured in 1-cm cell against a white standard with the MOMCOLOR colorimeter. • unripe fruit; x ripe fruit; △ over-ripe fruit. The values represent the averages of three replicates

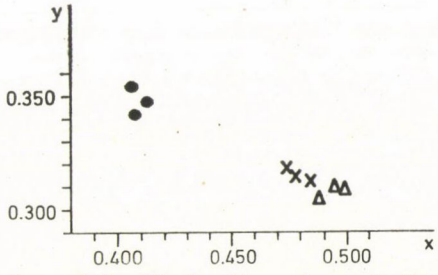

Fig. 2. Changes in the colour of the *Findus 27* raspberry fruits in the course of ripening. Experimental conditions and symbols as in Fig. 1

colour change when over-ripe (Fig. 1). The colour change of the *Findus 27* raspberry fruits is most marked between the half-ripe and ripe stages (Fig. 2), while the fruit colour of the *Knevett* variety changes more or less uniformly during the ripening process (Fig. 3). As seen from the data related to colour determination the hue (x) is more characteristic of the colour changes in certain raspberry varieties than the value (y), since the differences are lower in the latter.

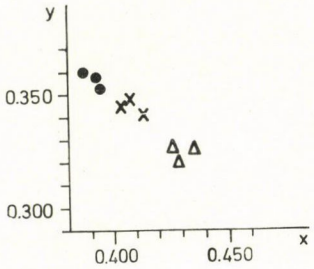

Fig. 3. Changes in the colour of the *Knevett* raspberry fruits in the course of ripening. Experimental conditions and symbols as in Fig. 1

2.2. pH changes during ripening

The differences between the pH values of unripe and over-ripe fruits amounted to not more than a few tenths. The average pH of unripe fruits was 3.0, that of ripe fruits 3.4 and of over-ripe ones 3.5, respectively.

2.3. Changes of the absorption spectrum in the course of ripening

The absorption spectra of the 0.1 *N* hydrochloric acid extracts of fruits in different ripening stages and belonging to the three raspberry varieties under investigation are shown in Figs. 4—6.

It can be seen that the maxima and shapes of the absorption curves do not depend on the ripening stage or on the colour changes characterizing the fruits of the individual varieties. The same was found for fruit juices diluted with distilled water, though there was a greater ripeness-dependent difference in the colour of these extracts than in the colour of the corresponding hydrochloric acid extracts, as could be observed by the naked eye, as well.

2.4. Changes in the anthocyanin ratios during ripening

Five anthocyanins were found in the fruit extracts of the investigated raspberry varieties (Fig. 7). These were, in the order of increasing R_f values: a cyanidin-3-glucoside derivative (*a*: $R_f = 0.14$), cyanidin-3-glucoside (*b*:

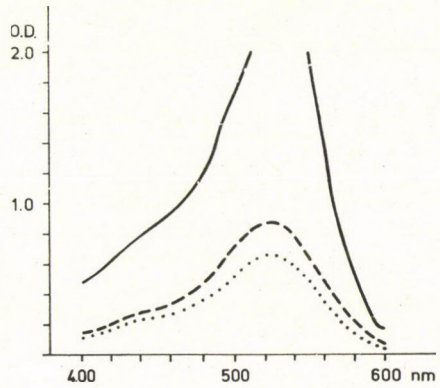

Fig. 4. Absorption curve of the fruit extract of the *Bolgarski Rubin* raspberry variety. 0.1 *N* hydrochloric acid extract (10 : 1), measured with the UNICAM SP 500 spectrophotometer in a 1-cm cell at 5 nm intervals. ..... unripe fruits; – – – ripe fruits; ——— over-ripe fruits

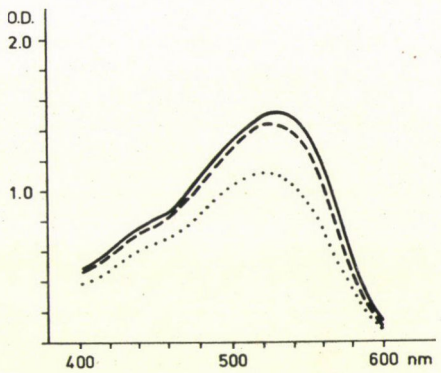

Fig. 5. Absorption curve of the fruit extract of *Findus 27* raspberry variety. Experimental conditions and symbols as in Fig. 4

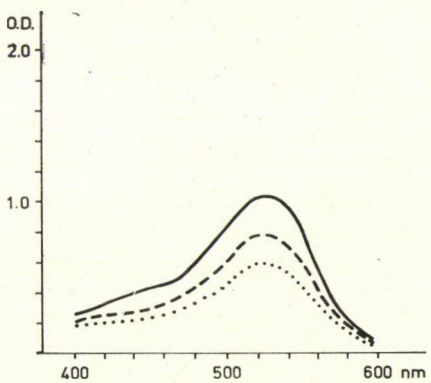

Fig. 6. Absorption curve of the fruit extract of *Knevett* raspberry variety. Experimental conditions and symbols as in Fig. 4

$R_f = 0.25$), cyanidin-3-rhamnoglucoside (*c*: $R_f = 0.32$), cyanidin-3-diglucoside (*d*: $R_f = 0.51$) and cyanidin-3-glucosyl rutinoside (*e*: $R_f = 0.76$). Although the ratio between the individual anthocyanins changes in dependence on the variety, this is affected by the ripening stage only insignificantly, as demonstrated on the fruits of the *Bolgarski Rubin* variety (Fig. 7). The same was found to be true for the other two raspberry varieties, too.

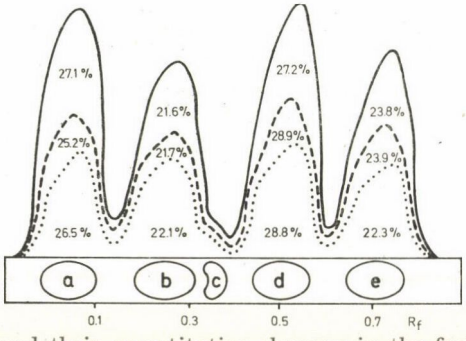

Fig. 7. Anthocyanins and their quantitative changes in the fruits of *Bolgarski Rubin* raspberry variety during ripening. Extract made with methanol containing 1 per cent of hydrochloric acid (10 : 1). After filtration, evaporated under N_2 on a 40 °C water bath, residue taken up in 1 ml of methanol containing 1 per cent hydrochloric acid. Fifteen μl applied on MN 214 paper, solvent: 5 per cent formic acid, run at room temperature in the dark. Densitometry with the ZEISS ERI-10 apparatus using integration. The figures in the peaks refer to the percentage quantities of anthocyanins. For the meaning of indices *a* to *e* see text

3. Conclusions

At first, we assumed that the deep purple colour of fruits of certain raspberry varieties when over-ripe might probably be associated with the formation of an anthocyanin–metal ion–polyphenol complex (GEISSMAN *et al.*, 1953; ASEN *et al.*, 1972). Namely, at pH values of 3.5 to 3.7 which correspond to that of over-ripe fruits, the absorption maximum of these complexes is at 570 nm, while in strongly acid media (below pH = 3.0) no complex is formed (ASEN *et al.*, 1969). The fact that under our experimental conditions, *i. e.* in 0.1 *N* hydrochloric acid, the expected shift of the absorption maximum was not observed, despite visible colour changes in the juices and fruits as confirmed by colour measurements, was attributed to the above observation. When, however, the absorption curves of juices made from fruits of different degrees of ripeness were recorded after dilution with distilled water and filtration, we again failed to obtain λ_{max} values at 570 nm. Hence, in the over-ripe state, that is in the deep purple fruits, an anthocyanin–metal ion–polyphenol complex cannot be made responsible for the development of undesirable colour. The existence of such a complex is also improbable for the reason that raspberries

contain citric acid even when over-ripe, and in the presence of this acid, no complex is formed (JURD & ASEN, 1966).

The stability of the absorption maxima (see Figs. 4 to 6) confirms the absence of chemical change in anthocyanins during ripening of the fruits. Moreover, as indicated by the combined method of paper chromatography and densitometry, the individual anthocyanins increase proportionally as the ripening proceeds. Consequently, there is no significant change in their ratios either (Fig. 7). Anyhow, no shift of the absorption maxima can be expected from changes in the ratio of individual anthocyanins, since raspberry fruits contain exclusively cyanidin glycosides, the λ_{max} values of which deviate by not more than a few nm from one another (HARBORNE, 1958; TANCHEV, 1971).

According to BAYER (1958) the blue colour of cornflower is due to the presence of a polysaccharide–metal ion complex. Theoretically, in raspberry fruits anthocyanins might also be linked to some polysaccharide. It is, however, unlikely that this would result in a considerable change of colour. In the cornflower, too, the blue colour is the result of binding of the metal ion to the cyanin molecule, and the polysaccharide carrier actually does not affect the absorption spectrum.

The characteristic deep purple colour of over-ripe fruits of certain raspberry varieties, such as of *Bolgarski Rubin*, *Lloyd George* or *Schönemann* might be caused by an "overproduction" of anthocyanins. In this respect, we refer to the classical work of WILLSTÄTTER and MALLISON (1915) in which they showed that blue cornflowers contain 0.05 to 0.07 per cent of cyanin (calculated on a dry matter basis), while in the petals of a dark purple variety the cyanin content might reach 13 to 14 per cent. Although we did not determine the absolute quantity of anthocyanins in raspberry fruits, comparison of Figs. 4, 5 and 6 clearly shows that in the over-ripe dark purple fruits of the *Bolgarski Rubin* variety the anthocyanin content is much higher than in fruits of the other two varieties, the fruits of which are still reddish-violet even when over-ripe.

The marked stability of the absorption maximum in hydrochloric acid makes possible a comparison between the anthocyanin content characteristic of various raspberry varieties by measuring the *O. D.* values at 525 nm. When plotting these values against the ripening stage (Fig. 8), the curve obtained will provide information on the rate of anthocyanin accumulation in the fruits of the investigated varieties.

As regards accumulation of colouring substances in the fruits, the following conclusions may be drawn for the raspberry varieties studied:

— in the *Knevett* fruits, the content of colouring substances increases more or less uniformly during ripening;

— in the fruits of *Findus 27* variety the increase in colouring substance content is confined to the first half of ripening while there is practically no change in the second half of the process;

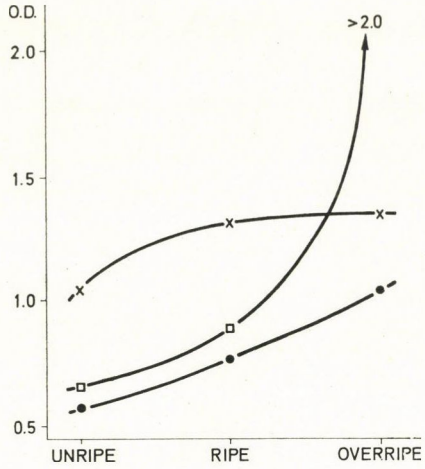

Fig. 8. Changes in the anthocyanin contents of the fruits of the investigated raspberry varieties during ripening. Values measured at the absorption maximum of 525 nm (optical density, see Figs. 4 to 6) are plotted *vs.* the stage of ripening. —□—□— *Bolgarski Rubin;* —x—x— *Findus 27;* — · — · — *Knevett*

— in the fruits of the *Bolgarski Rubin* raspberry, the amount of colouring substances shows an extraordinarily great increase in the second half of ripening.

These findings permit the selection of raspberry varieties and hybrids in the fruits of which, despite a higher anthocyanin content, the colour changes are relatively slight during ripening. This property is of great economic importance, since only by growing of such raspberry varieties can a uniform colour of the fruits at harvest be achieved, independently of the stage of ripeness of individual fruits.

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The authors wish to thank Mr. Gyula LUKÁCS (Hungarian Optical Works) for his useful suggestions and for placing the various fittings of the MOMCOLOR apparatus at their disposal.

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A COMPARATIVE STUDY OF THE POLYOL–SUGAR INTERCONVERTING ACTIVITY OF VARIOUS YEAST SPECIES, IN RELATION TO THE INTERPRETATION OF THE MECHANISM OF TAXONOMICAL UTILIZATION TESTS

E. K. Novák, A. S. El Nawawy and L. Katona

(Received June 7, 1973)

The enzymic polyol–sugar redox interconverting activities of some yeast species were studied. With the 18 strains examined so far it was found that out of the six D-mannitol non-assimilators only two were cryptic, *i.e.*, able to dehydrogenate this substrate in cell- free extracts.

In detailed studies carried out with five species (one strain of each) cryptic ity of some taxonomic characters was frequently found, *i.e.*, the redox pattern was broader than the utilization one. The possible bases of this phenomenon — the broad specificity of the redox enzymes, and lack of transport or the metabolic reaction following the redox one — were discussed. The participating enzymes were grouped on the basis of the chemical and biochemical nature or type of the sugar-polyol interconversion. Four new redox activities (reduction of L-xylose, D-arabinose, D-lyxose and D-mannose with NADP · H), previously not known in yeasts, were also detected.

In bacteria direct enzymic isomerization is the main if not the only interconversion reaction between aldoses and ketoses (Anderson & Wood, 1962; Axelrod, 1960; Burma, *et al.*, 1957; Camyre & Mortlock, 1965; Cohen, 1953; Doudoroff, 1961), however, they have also dehydrogenating enzymes acting in the utilization of polyols. On the contrary with yeasts the sporadic results to date reflect on another pathway in ketose–aldose interconversions, *i. e.*, through polyol intermediates by means of oxidoreductases (Barnett, 1968a). Thus the sugar–polyol interconversions in yeasts are significant from many aspects. Besides being of technological importance (Spencer, 1968) they are interesting also from the point of view of biochemistry and physiology of yeasts. Moreover, greater attention must be paid to them in the taxonomy in connection with the biochemical interpretation of the results of physiological identification test (Novák, 1968; Novák & Deák, 1967, 1970; Novák *et al.*, 1973). According to data known to date the enzymes of these processes, the oxidoreductases — polyol-dehydrogenases or aldose-(aldol) and ketose-(ketol) reductases — are of broad specificity and thus numerous correlations in sugar source utilization may be due to them or more exactly to their group specificity (Barnett, 1966; Barnett, 1968a, b). These correlations are characteristic for nearly all of the yeast species, however, some exceptions are known (Novák & Deák, 1967, 1970; Novák, *et al.*, 1973). When a correlation rule, deduced from the summarized carbohydrate utiliza-

tion data of the "yeast kingdom" proves to be invalid for an individual species or strain, two assumptions can be made. Either the individual enzymes of the oxidoreductase set of the various yeast species are of different specificity (the "same" enzyme of various yeasts does not have the same substrate spectrum), or it is not the oxidoreductases but some block in metabolism (before the redox reactions as the transport, or after it as *e. g.* the following phosphorylations step) that causes the anomaly in the correlative substrate utilization. As to sugar transport in a comparative study (DEÁK & NOVÁK, 1971; NOVÁK & DEÁK, 1972), there again group specificity was found, and thus the necessity of a comparative oxidoreductase-activity investigation became obvious.

1. Materials and methods

Enzyme extracts were prepared from the following yeasts:*

1. *Endomyces reessii* 580
2. *Dekkeromyces wickerhamii* (Phaff)
3. *Geotrichum candidum* 228/1966
4. *Geotrichum candidum* 484/1960
5. *Geotrichum candidum* K91/1968
6. *Geotrichum candidum* K92/1968
7. *Geotrichum hirtum* 630
8. *Geotrichum linkii* 22/1960
9. *Geotrichum linkii* 65/1965
10. *Geotrichum loubieri* CVII/1968
11. *Geotrichum matalensis* 92—II/1958
12. *Procandida albicans* 85/1957
13. *Procandida tropicalis* 302/1964
14. *Candida utilis* NRRL Y—900
15. *Candida pelliculosa* (El-Nawawy 1969)
16. *Candida lipolytica* NRRL Y—1094
17. *Procandida tropicalis* NRRL Y—1410
18. *Rhodotorula gracilis* NRRL Y—1091

The strains were kept on molasses agar (CSILLAG, 1950) in our collection. In the preliminary experiments the strains Nos. 1—13 were propagated in Roux bottles for 72 hours at 26°C on carbon source agar (LODDER & KREGER-VAN RIJ, 1952) containing 2% sorbitol and supplemented with 10% liquid yeast extract (NOVÁK *et al.*, 1967). In the detailed experiments with the remaining strains (Nos. 14—18) a synthetic liquid medium (DEÁK & KOTYK, 1968) was used with a carbon source of 2% glucose or xylose, and 0.5% liquid

* Names of taxa according to NOVÁK and ZSOLT (1961), while for *G. hirtum* and *G. loubieri* see MORENZ (1964).

yeast extract as vitamin source, added before inoculation. Cultivation was performed in volumes of 80 ml in 500-ml Erlenmeyer flasks at 22°C for 48 hours on a rotary shaker (300 rpm., 4 cm stroke).

In both cases at the end of the incubation period the cells were harvested by centrifugation, washed three times with distilled water and disintegrated at 4°C with quartz sand. The enzymes were extracted thereafter with M/15 pH = 7.5 phosphate buffer (calculating 1.25 ml buffer for every g of fresh wet cells) and were either used immediately as in the preliminary experiments or after an overnight dialysis as in the detailed ones.

Chemicals originated from the following sources: NAD and NADP *Reanal* (Hungary), NAD·H. *Pabst Laboratories* (Illinois), NADP·H. *Boehringer and Sons* Ltd (FRG), xylitol* and D-arabinitol *International Biochemical Corp.* (Ohio), D-lyxose and D-galactose *Fluka* A. G. (Switzerland), L-xylose *Dr Theodor Schuchardt* GmbH (FRG), L-mannose *Calbiochem* (California), D-tagatose *Koch-Light Laboratories* (England), all other sugars and polyols *Reanal* (Hungary).

Assay procedure was as follows: into 1-cm photometric quartz cuvettes 0.6 ml enzyme extract (of appropriate pH), 0.9 ml 0.1 molar substrate (in appropriate phosphate buffer) was pipetted and the optical density was measured against a blank containing only buffer and enzyme. The reaction was thereafter started by adding 0.6 mg coenzyme in 0.3 ml volume. In the endogenous control, buffer was substituted for substrate. The reaction was followed for 30 minutes in a *Specol* (Carl Zeiss, Jena) spectrophotometer at 365 nm. Using polyol substrates and oxidized coenzymes the pH was 9.0, while with sugars and reduced coenzymes 7.1. To adjust the appropriate pH in enzyme extracts they were diluted to 1 : 1 with the respective concentrated phosphate buffer.

Assimilation of the compounds was determined according to the standard auxanographic technique (LODDER & KREGER-VAN RIJ, 1952).

2. Results and conclusions

2.1. Preliminary experiments

Primarily it was interesting to know, whether the individual lack of positive correlation, wide spread among yeasts, between the dSt and dMt utilization (NOVÁK & DEÁK, 1967, 1970; NOVÁK *et al.*, 1973) is dependent on

* Abbreviations or symbols of substrates: D- and L-arabinose = **dA** and **lA**, D- and L-arabinitol = **dAt** and **lAt**, D- and L-fructose = **dF** and **lF**, galactitol = **Gt**, D- and L-galactose = **dG** and **lG**, D- and L-glucose = **dD** and **lD**, D- and L-lyxose = = **dLy** and **lLy**, D- and L-mannitol = **dMt** and **lMt**, D- and L-mannose = **dMn** and **lMn**, ribitol = **Rt**, D- and L-ribose = **dR** and **lR**, D- and L-ribulose = **dRu** and **lRu**, D- and L-sorbitol (glucitol) = **dSt** and **lSt**, D- and L-sorbose = **dSo** and **lSo**, D- and L-tagatose = **dTg** and **lTg**, xylitol = **Xt**, D- and L-xylose = **dX** and **lX**, D- and L-xylulose = **dXu** and **lXu**.

the lack of the respective dehydrogenases or of the transport systems. Therefore yeast species positive for both (Nos. 5, 6, 10, 12 and 13), or only for dSt assimilation (Nos. 1—4, 7—9, 11) were investigated. It is to be mentioned here that a similar problem — the coincidence of dMt and dSt (and Gt resp.) utilization and its biochemical basis — was studied also by other authors (SEBEK & RANDLES, 1952; SHOCKLEY & RANDLES, 1954), however in bacteria.

In species assimilating both polyols, both dehydrogenating activities could be detected (Nos. 5, 6, 10, 12, 13), although the values of activities were

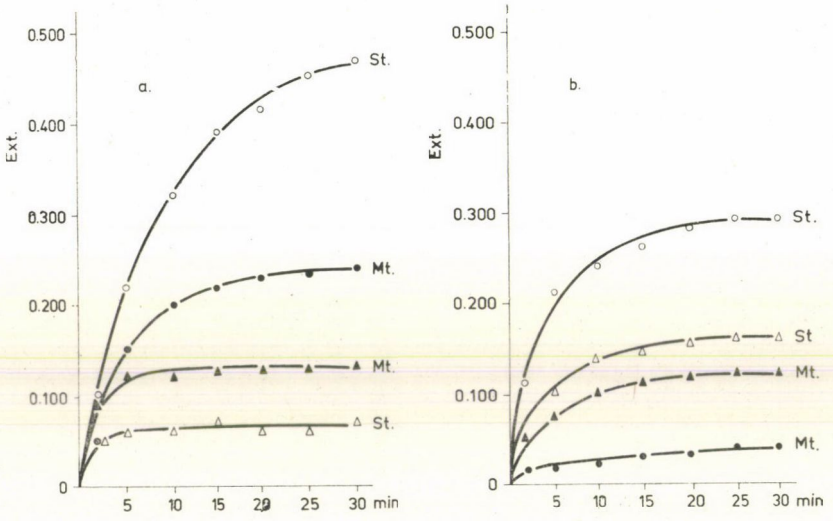

Fig. 1. Types of NAD dependent D-sorbitol and D-mannitol dehydrogenating activities with various yeasts. a: ○—○ and ●—● = *E. reessii* dSt and dMt dehydrogenation; △—△ and ▲—▲ = *G. loubieri* dSt and dMt dehydrogenation. b: ○—○ and ●—● = *D. wickerhamii* dSt and dMt dehydrogenation; △—△ and ▲—▲ = *G. candidum* K92/1968 dSt and dMt dehydrogenation

not the same with dSt and dMt. Except for *G. loubieri* the dSt dehydrogenation proved to be stronger. This may be caused by the fact that out of the two probably present dSt dehydrogenating enzymes (see later) only one is able to react also with dMt.

In contrast to this, out of the dMt non-assimilating species some were able to dehydrogenate this substrate (Nos. 1, 7), while others were not or only to a negligible extent (Nos. 4, 9, or 2, 3, 8, 11, resp.).

The above results are summarized in Fig. 1 and Table 1.

The dMt dehydrogenating activity found with dMt non-assimilating species refers to a transport defect, the possibility of which was mentioned also by BARNETT (1966, 1968a). The post-oxidation block, however, is improbable here as the obligate product (dF) of dMt oxidation would have been further metabolized if produced.

Table 1

Assimilation and dehydrogenation of D-sorbitol and D-mannitol by various yeast species

Serial number and name of species	D-sorbitol		D-mannitol	
	assimilation	dehydrogenation*	assimilation	dehydrogenation*
1. *E. reessii*	(+)	++	—	++
2. *D. wickerhamii*	(+)	++	—	((+))
3. *G. candidum*	+	+	—	((+))
4. *G. candidum*	+	++	—	—
5. *G. candidum*	+	++	+	(+)
6. *G. candidum*	+	+	+	+
7. *G. hirtum*	+	++	—	(+)
8. *G. linkii*	((+))	+	—	((+))
9. *G. linkii*	((+))	+	—	—
10. *G. loubieri*	(+)	(+)	+	+
11. *G. matalensis*	+	++	—	((+))
12. *Pc. albicans*	+	+	+	+
13. *Pc. tropicalis*	+	+	+	+

ASSIMILATION	DEHYDROGENATION (ΔE)
++ = strong	>0.20
+ = medium	0.10—0.20
(+) = weak	0.05—0.10
((+)) = very weak	0.02—0.05
— = negative	<0.02

* Measured in cell-free extract with NAD coenzyme (see methods).

It is worth mentioning that *Pc. albicans* showed a "+" Rt dehydrogenating activity with NAD coenzyme, which agrees well with data of the literature (CHAKRAVORTY *et al.*, 1962; VEIGA *et al.*, 1960). Moreover, *Pc. tropicalis* substantially dehydrogenated Rt with both NAD and NADP, a fact that corresponds to assimilation data (LODDER, 1970).

2.2. Detailed studies

The polyol–sugar interconversions measured with NAD/NAD·H or NADP/NADP·H supplemented cell-free extracts of the investigated strains of *C. utilis*, *Pc. tropicalis*, *Rhodotorula gracilis*, *C. lipolytica* and *C. pelliculosa* are summarized in Figs. 2—6, while Table 2 contains the carbon source assimilation data of these strains relative to the substrates used in the enzymological reactions, and also the sugar transport characteristics of these strains based partially on the work of NOVÁK and DEÁK (DEÁK & NOVÁK, 1971; NOVÁK & DEÁK, 1972).

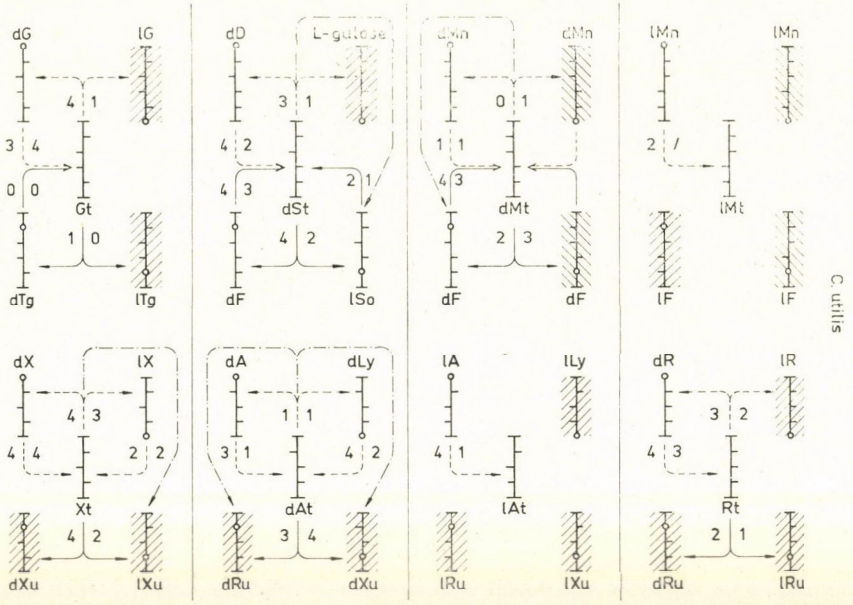

Fig. 2. Polyol–sugar interconverting activites of *C. utilis* cell-free extracts. NAD and NAD · H reactions: →; NADP and NADP · H reactions: — — → (towards aldoses), or — · → (towards ketoses). Numbers on the left side of the vertical part of the arrows refer to the activity of the cell-free extracts of cells, grown on dX while on the right side to that of cells grown on dD. 4: $\Delta E > 0.200$; 3 : $\Delta E = 0.200 - 0.100$; 2 : $\Delta E = 0.100 - 0.050$; 1 : $\Delta E = 0.050 - 0.010$; 0 : $\Delta E < 0.010$. ////: Covered substrates were not studied or identified. \\\\: Covered substrates are not relevant as they are the same as on the left side (duplicates)

Generally, as it is seen, all the five species lacked dTg-NAD · H reaction. On the contrary, they reduced NAD with dSt, dMt, Xt and Rt, and oxidized NAD · H with lSo and dF. Similarly, the NADP reduction with dSt, Xt, Rt, Gt and the NADP · H oxidation with dD, dX, dA, dLy, dR, dG, lA, were general.

In many cases the cultivation on dX enhanced the activities or initiated new ones (as compared to cultivation on dD). This is very interesting for us with respect to the Gt or dA metabolism because in our earlier work we found a statistical correlation between the dX and Gt or dA utilization of the yeast species taxonomically accepted (NOVÁK & DEÁK, 1967, 1970; NOVÁK *et al.*, 1973) without any reasonable biochemical explanation in the former case.

Frequently the crypticity of some "taxonomic" characters was observed which is to be considered as the consequence, on the one hand, of the broad specificity of the carbohydrate interconverting enzymes acting in the metabolism of "utilized" substrates (causing the positive redox reaction by non-

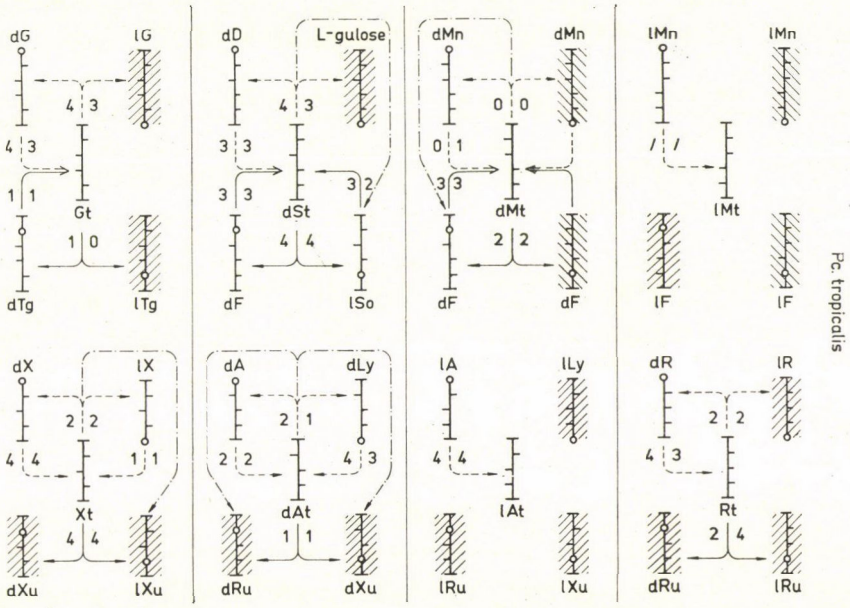

Fig. 3. Polyol–sugar interconverting activities of *Pc. tropicalis* cell-free extracts. (For further explanation see Fig. 2.)

utilized ones) and, on the other hand, of the lack of transport or of the metabolic reactions following the redox one (making utilization impossible). *E.g. C. pelliculosa* does not utilize dR, dA and Gt, although it contains the enzymes for their transformation.

To make the further detailed discussion more expedient the reactions are systematized according to the type of the sugar partner (ketose or aldose) and, within these groups, according to the molecular configurations involved (Figs. 7 & 8) — the use of configuration for the characterization of this type of reaction originates from BERTRAND (*Bertrand-Hudson* rule — BERTRAND, 1898 a, b, 1904; HANN *et al.*, 1938). Moreover, the literature data concerning the polyol–sugar interconverting enzymes are summarized (Table 3).

Figs. 9 and 10 represent a theoretical interconversion scheme based on the published reactions of the substances in question.

Prior to the discussion of the results of the individual species investigated we have to call the attention to some main problems of the above summarizations. There are some contradictions concerning the specificity of some enzymes — *e.g.* lIdt : NAD-oxidoreductase is considered by some authors to be active on Gt, while inactive by others (see Table 3), however Gt : NAD--oxidoreductase is mentioned as active on every "L-threo"-substrate, *etc.* —

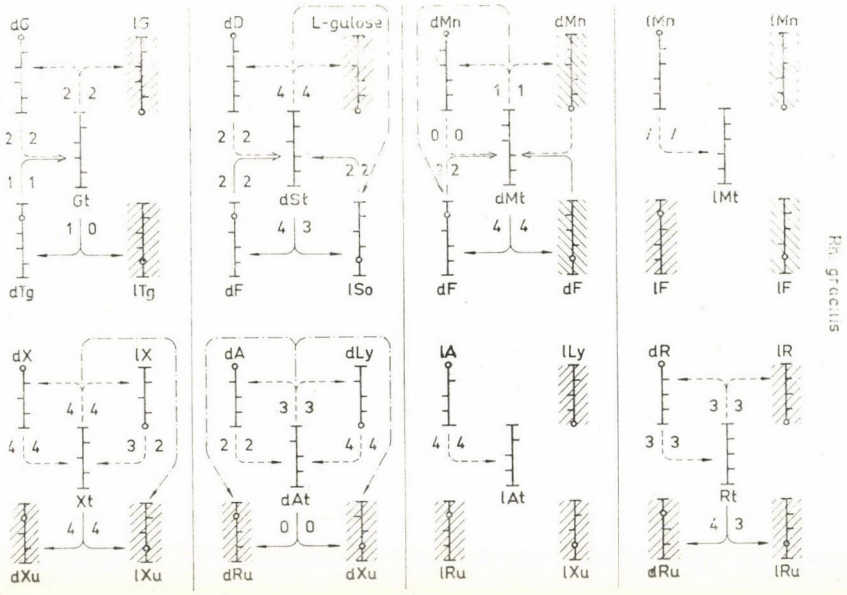

Fig. 4. Polyol–sugar interconverting activities of *Rh. gracilis* cell-free extracts. (For further explanation see Fig. 2.)

this justifies the present summarizing classification; and alditol:NADP--oxidoreductase seems to be active not only in configuration group 1 but also in group 2, however, to a lesser degree (see Fig. 8 and Table 3).

2.2.1. C. utilis. According to the literature this species contains all the four polyol–ketose interconveting NAD-enzymes (Table 3), however, in our experiments, at least after cultivation on dX, all the deliminative reactions were found except dTg reduction. The missing of Gt oxidation corroborates the assumption found in the literature that lIdt : NAD-oxidoreductase is not able to activate Gt oxidation. Exactly an enzyme of this type was found in *C. utilis* (Table 3). Thus our strain contains at least two enzymes, dMt : NAD--oxidoreductase and lIdt : NAD-oxidoreductase, sufficient to explain the activities found with NAD/NAD·H.

C. utilis was published to contain the only one known of the four theoretical types of polyol–aldose interconverting enzymes, the alditol-NADP--oxidoreductase. All the activities referring to the presence of this enzyme were found by us. Besides these, however, some other activities were demonstrated, too. Reactions of lMn and dR/Rt are explained by the not rigorous sensitivity of the enzyme to the configuration of the second hydroxyl of the substrate. In other words, the activity depends qualitatively on the configuration of the first and only quantitatively on that of the second hydroxyl (see also Table

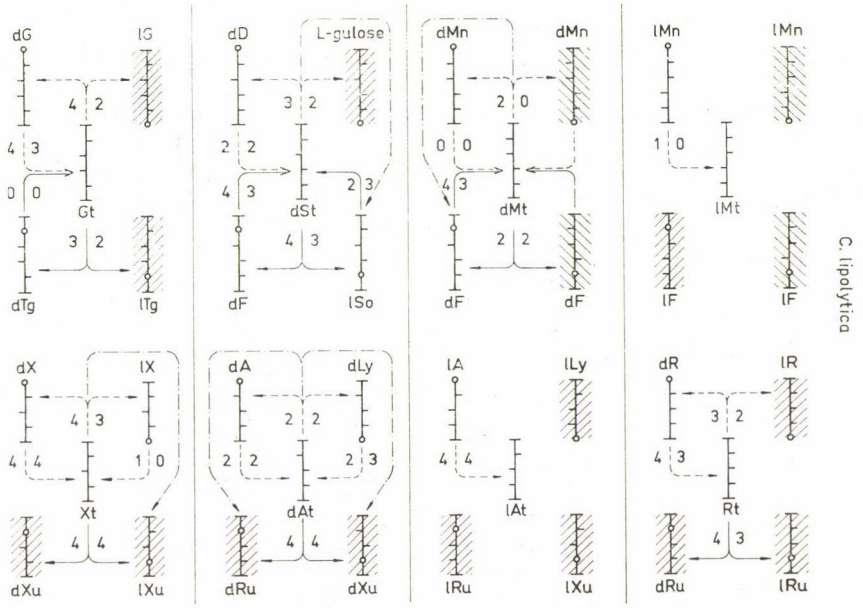

Fig. 5. Polyol–sugar interconverting activities of *C. lipolytica* cell-free extracts. (For further explanation see Fig. 2.)

3). In the case of dMt/dMn and dAt/dLy the presence of another enzyme must be supposed (*cf.* Fig. 8). The assumed presence of dMt : NADP-oxidoreductase and/or Xt : NADP-oxidoreductase would explain only the dehydrogenation of dMt and dAt, but not the opposite reactions (these enzymes are ketose producing ones, see Table 3). The presence of a third enzyme (Fig. 8) is also probable which would explain the reactions of Xt/lX and dAt/dA. These again are not the substrates of alditol : NADP-oxidoreductase (Table 3), and similarly Xt : NADP-oxidoreductase can not account for these reactions.

It is clear that the questions raised above need further detailed biochemical investigations, but let us turn to their interrelations with utilization and transport.

The negative lSo utilization, may have, as the transport is operating, a quantitative explanation, because cells cultivated on dD have only weak activity in lSo–dSt–dF or lSo–dSt–dD reactions (which would lead to main intermediary routes), and the other known product of lSo reduction, the lIdt has no further known metabolic reaction. The negative lA, lX and dR utilization is explained by the poor transport. In the case of dA and dLy, as the transport is operating, the poor activity of the initial transformation step may

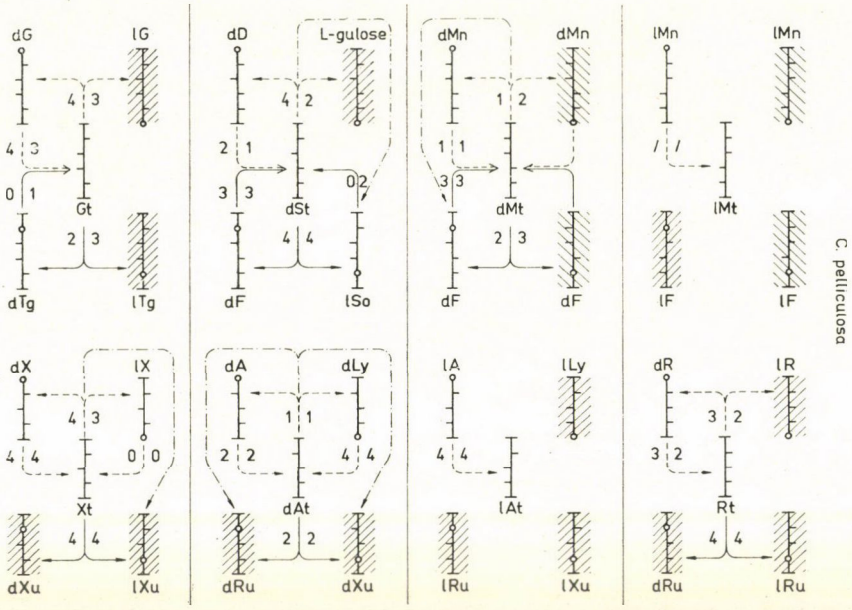

Fig. 6. Polyol–sugar interconverting activities of *C. pelliculosa* cell-free extracts. (For further explanation see Fig. 2.)

account for the negative utilization. The dG, although it is transported well, cannot be utilized, as on the one pathway the second step (at reduction with NAD) is missing, and on the other pathway at least one member of the waldenase complex is probably not present.

2.2.2. Pc. tropicalis. On the basis of our results (as literature data were not found) this yeast may have at least the following polyol–ketose interconverting enzymes: dMt : NAD-oxidoreductase and lIdt : NAD-oxidoreductase (with no activity in Gt-dTg reaction).

This species contains, according to the literature, the alditol : NADP-oxidoreductase, which was confirmed by us, however, some other activities were also found. Although weak, they suggested the presence of the other two enzymes mentioned with *C. utilis*.

The lack of lX and dR utilization may be caused by poor transport (and by the poor reduction of the former). The dA and lA, as their transport and reduction are satisfactory, may have a metabolic block in further reactions. The Gt, as it is able to transform to dG, must have a transport block.

2.2.3. Rh. gracilis. The results concerning the polyol–ketose reactions (literature data are not known) were similar to those of the above two species, only dAt oxidation was hardly demonstrable. Thus the enzyme set is similar to those of the above two species.

Table 2

Sugar and polyol assimilation and sugar transport of the five yeasts studied

Substrates	*Rh. gracilis*		*C. pelliculosa*		*C. utilis*		*C. lipolytica*		*Pc. tropicalis*	
	Ass.	Tr.	Ass.	Tr.	Ass.	Tr.	Ass.	Tr.	Ass.	Tr.
dTg	/	/	/	/	/	/	/	/	/	/
dD	+	a	+	a	+	a	+	a	+	a
dF	+	a	+	a	+	a	+	a	+	a
dMn	+	a	+	a	+	a	+	a	+	a
dG	(+)	A	+	A	−	A	+	A	+	a
lSo	+	a	+	A	−	A	+	A	+	a
lMn	/	/	/	/	/	/	/	/	/	/
dX	((+))	A	+	a	+	A	+	a	+	a
dA	−	A	−	A	−	A	−/+	A	−	A
lA	−	A	(+)	HA	−	W	+	A	−	HA
dR	−	H	−	H	−	H	−	H	−	W
dLy	−	/	(+)	/	−	A	−	/	((+))	A
lX	−	T	−	HT	−	HT	−	T	−	HT
dMt	+/−		+		(+)/−		(+)		+	
dSt	+/−		+		−		−		+	
Gt	−/+		−		−		−		−	
Rt	+/−		+/−		−		−		−	
dAt	/		/		/		/		/	
Xt	/		/		/		/		/	

+ = positive; (+) = weak; ((+)) = very weak or weak and latent; − = negative; +/− = positive but rarely negative; −/+ = negative but rarely positive; / = not studied. a = active, uphill transport with metabolization; A = as before but without metabolization; t = facilitated diffusion (downhill transport) into the total sugar space, with metabolization; T = as before but without metabolization; h = facilitated diffusion into the half sugar space, with metabolitzation; H = as before, but without metabolization; W = weak transport (steady state level is less than the diffusion equilibrium for the half sugar space). Interpretation of transport types, see in ref. NOVÁK & DEÁK (1972)

As to polyol–aldose reactions the presence of alditol : NADP-oxidoreductase found by others was confirmed, however, some activities referred to the presence of the other two enzymes mentioned with the above two species.

The negativity of lX and dR utilization may have its explanation in the poor transport, while that of dA, dLy and lA, as their transport and reduction are satisfactory, in the consecutive reactions.

2 2.4. C. lipolytica and C. pelliculosa. These were similar in every respect, with only negligible quantitative differences.

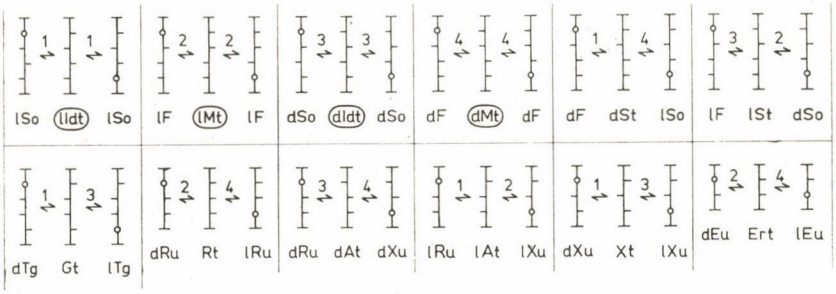

Fig. 7. Types of polyol–ketose interconversions. When polyol is oxidized to ketose the reacting hydroxyl is the distal one of a secondary hydroxyl pair adjacent to one of the two primary hydroxyls of the molecule. According to the steric configuration of the hydroxyl pair in projection at the bottom there are four possibilities: 1 = L-threo: reaction 1, type lIdt dehydrogenation to lSo (see also the similar ones); 2 = L-erythro: reaction 2, type lMt dehydrogenation to lF (see also the similar ones); 3 = D-threo: reaction 3, type dIdt dehydrogenation to dSo (see also the similar ones); 4 = D-erythro: reaction 4, type dMt dehydrogenation to dF (see also the similar ones)

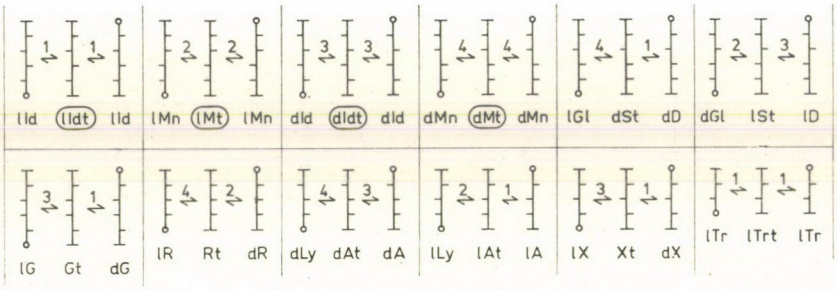

Fig. 8. Types of polyol–aldose interconversions. When the polyol is oxidized to aldose the reacting hydroxyl is one of the two primary ones and the adjacent secondary hydroxyl pair may have four steric configurations (the primary in question being at the bottom): 1 = L-threo: reaction 1, type lIdt dehydrogenation to lId (see also the similar ones); 2 = L-erythro: reaction 2, type lMt dehydrogenation to lMn (see also the similar ones); 3 = D-threo: reaction 3, type dIdt dehydrogenation to dId (see also the similar ones); 4 = D-erythro: reaction 4, type dMt dehydrogenation to dMn (see also the similar ones)

Compared to the previous three species the presence of Gt reduction with NAD was a new finding here. Thus the smallest enzyme set which can be proposed here contains dMt : NAD-oxidoreductase and dIdt : oxidoreductase.

Concerning the polyol–aldose reactions, the presence of alditol : NADP--oxidoreductase is evident. In relation to the other two enzymes demonstrated with more or less probability in the previous species, some contradictory results were found here. The reactions of dAt, dLy and dA refer to the presence, while those of dMn, dMt and lX to the lack of these enzymes.

The lack of Gt and dR utilization by both species originates from the lack or poor activity of transport, as their redox reactions are good. The

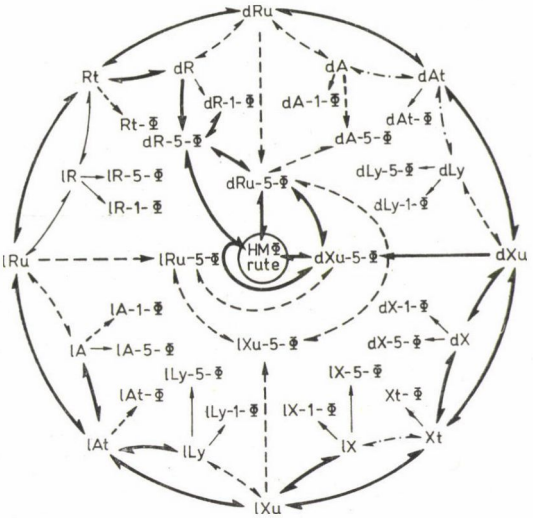

Fig. 9. Intermediary metabolism of C_5 carbohydrates. ——— = Biochemically probable; — — — — = demonstrated in living organisms; ▬▬▬ = demonstrated in yeasts; — · — · — · — = demonstrated by us in yeasts (new ones). Φ-phosphate group. (On the basis of literature data as cited in the text, and ref. FLORKIN & STOTZ, 1965; MCCORKINDALE, 1953; TOMOEDA & HORITSU, 1969)

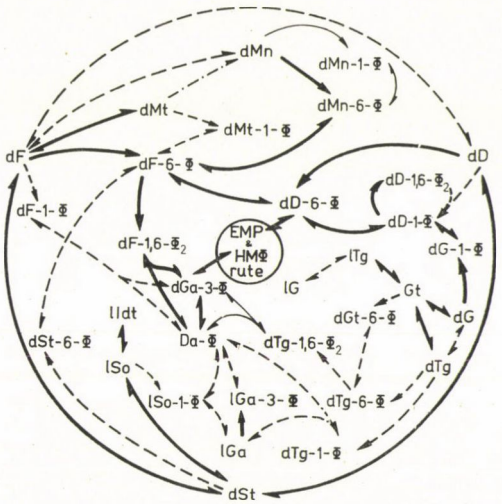

Fig. 10. Intermediary metabolism of C_6 carbohydrates. ——— = Biochemically probable; — — — — = demonstrated in living organisms; ▬▬▬ = demonstrated in yeasts; — · — · — · — = demonstrated by us in yeasts (new ones). (On the basis of literature data as cited in the text, and ref. BURNS *et al.*, 1955; FLORKIN & STOTZ, 1965; HORWITZ, 1966a; KIRCHHOF, 1962; MCCORKINDALE, 1953)

Table 3

Systematization of polyol: sugar-oxidoreductases

class: A., polyol: ketose interconversion.
form: 1., L-threo-diol: ketol-oxidoreductase, *group:* a., NAD-enzymes, *type:* Idt :NAD-oxidoreductase (EC. 1.1.1.14.).*

Organism	Demonstrated reaction	References
Candida albicans	dSt—dF, Xt—dXu, lIdt—lSo,	(CHAKRAVORTY *et al.*, 1962; VEIGA *et al.*, 1960**)
Candida utilis	dSt—dF, Xt—dXu, lIdt—lSo,	(ARCUS & EDSON, 1956**; BARNETT, 1968b; CHAKRAVORTY & HORECKER, 1966; CHAKRAVORTY *et al.*, 1962; HORITSU & TOMOEDA, 1966)
Geotrichum candidum	dSt—dF, Xt—dXu,	(WAN & CHANG, 1965)
Hansenula anomala	dSt—dF, Xt—dXu, lIdt—lSo, Gt—dTg, lAt—lRu,	(SHAW, 1956***)
Pichia membrenae-faciens	dSt—dF, Xt—dXu, lIdt—lSo,	(BARNETT, 1968b)
Saccharomyces acidi-faciens	dSt—dF, Xt—dXu, lIdt—lSo,	(BARNETT, 1968b)
Saccharomyces mellis	dSt—dF, Xt—dXu, Gt—dTg,	(WEINBERG, 1962)
Saccharomyces rouxii	Xt—dXu, lAt—lRu,	(INGRAM & WOOD, 1965, 1966)
Torulopsis candida	dSt—dF, Xt—dXu, lIdt—lSo,	(BARNETT, 1968b)
Penicillium chryso-genum	dSt—dF, Xt—dXu, lAt—lRu,	(CHIANG & KNIGHT, 1959, 1960b, 1966; CHIANG *et al.*, 1958)
Acetobacter suboxydans	dSt—dF, Xt—dXu, lIdt—lSo,	(ARCUS & EDSON, 1956; CHAKRAVORTY & HORECKER, 1966; CHAKRAVORTY *et al.*, 1962; CUMMINS *et al.*, 1957a, b; KERSTERS & DE LEY, 1966)
Aerobacter aerogenes	Xt—dXu,	(ANDERSON & WOOD, 1962; FOSSITT *et al.*, 1964)
Bacillus subtilis	dSt—dF, Xt—dXu, lIdt—lSo,	(HORWITZ, 1966b)
Pseudomonas spp.	dSt—dF, Xt—dXu, lIdt—lSo, Gt—dTg, lAt—lRu,	(SHAW, 1962)
mammalian organs	dSt—dF, Xt—dXu, lIdt—lSo, lAt—lRu,	(BLAKELY, 1951; BRENSCH, 1943; CHAKRAVORTY & HORECKER, 1966; EDSON, 1953, 1961; KING & MANN, 1966; MCCORKINDALE & EDSON, 1953, 1954; MCNAIR SCOTT, 1961; TOUSTER & MONTESI, 1962; TOUSTER *et al.*, 1956; WILLIAMS-ASHMAN & BANKS, 1954**)

* enzyme code number is given if known, Xt:NAD-(EC.1.1.1.9), lAt:NAD-(EC.1.1.1.13), and Gt:NAD-oxidoreductase (EC.1.1.1.16.) is to be systematized here.
** referred to as inactive on Gt; contradictory data due to dMt:NAD--oxidoreductase, EC.1.1.1.67; (see form 4) contamination.
*** named as Gt-dehydrogenase to emphasize its activity in Gt-dTg reaction in contrast to *Acetobacter suboxydans* and liver enzyms, resp.

Table 3 (continued)

form: 2., L-erythro-diol:ketol-oxidoreductase, *group:* a., NAD-enzymes, *type:* lMt:NAD-oxidoreductase*.

Organism	Demonstrated reaction	References
Candida albicans	Rt—dRu,	(CHAKRAVORTY *et al.*, 1962)
Candida utilis	Rt—dRu	(BARNETT, 1968b; CHAKRAVORTY *et al.*, 1962; MCCORKINDALE & EDSON, 1954)
Geotrichum candidum	lAt—lXu	(MORET & SPERTI, 1962; WEINBERG, 1962)
Saccharomyces acidifaciens	lAt—lXu,	(BARNETT, 1968b)
Saccharomyces mellis	Rt—dRu, lAt—lXu,	(MORET & SPERTI, 1962; WEINBERG, 1962)
Torulopsis candida	Rt—dRu,	(BARNETT, 1968b)
Penicillium chrysogenum	lAt—lXu.	(CHIANG & KNIGHT, 1959, 1960a,b, 1966; CHIANG *et al.*, 1958)
Acetobacter suboxydans	Rt—dRu, lMt—lF, lAT— lXu,	(KERSTERS & DE LEY, 1966)
Aerobacter aerogenes	Rt—dRu, lAt—lXu, Ert— Eu,	(FOSSITT & WOOD, 1966; FROMM, 1958, 1959; FROMM & NELSON, 1962; JAKOBY, 1966; MORTLOCK *et al.*, 1965; NORDLIE & FROMM, 1959)

* lAt:NAD- (lXu forming — EC. 1.1.1.12) and Rt:NAD-oxidoreductase (EC. 1.1.1.56.) are to be systematized here.

form: 3., D-threo-diol:ketol-oxidoreductase, *group:* a., NAD-enzymes, *type:* dIdt:NAD-oxidoreductase.

Organism	Demonstrated reaction	References
Candida utilis	Xt—lXu, dAt—dRu,	(BARNETT, 1968b)
Hansenula anomala	Xt—lXu, dAt—dRu, dIdt—dSo, Gt—lTg, lSt—lF,	(SHAW, 1956)
Pichia delftensis	Xt—lXu, dAt—dRu,	(BARNETT, 1968b)
Torulopsis candida	X—lXu, dAt—dRu,	(BARNETT, 1968b)
Schizophyllum commune	Xt—lXu,	(NIEDERPRUEM *et al.*, 1965)
Aerobacter aerogenes	Xt—lXu,	(ANDERSON & WOOD, 1962)
Pseudomonas fluorescens	Xt—lXu, dAt—dRu, dIdt—dSo, Gt—lTg, lSt—lF,	(SHAW 1956, 1962)

group: b., NADP-enzymes, *type:* dIdt:NADP-oxidoreductase*.

Saccharomyces acidifaciens	Xt—lXu,	(BARNETT, 1968b)
Saccharomyces mellis	Xt—lXu, dAt—dRu	(WAN & CHANG, 1965)
Saccharomyces rouxii	dAt—dRu,	(INGRAM & WOOD, 1965, 1966)
Torulopsis candida	Xt—lXu,	(BARNETT, 1968b)
Penicillium chrysogenum	Xt—lXu,	(BYGRAVE & SHAW, 1964)
Aerobacter aerogenes	Xt—lXu,	(FOSSITT *et al.*, 1964)
Gluconobacter oxydans (Acet. subox.)	Xt—lXu.	(KERSTERS & DE LEY, 1966)
mammalian organs	Xt—lXu,	(HERS, 1957, 1960a, b; HICKMAN & ASHWELL, 1959; HOLLMAN & TOUSTER, 1956, 1957; MCNAIR SCOTT, 1961; TOUSTER & MONTESI, 1962; TOUSTER *et al.*, 1956**)

* Xt:NADP-oxidoreductase (EC. 1.1.1.10.) is to be systematized here.
** mentioned as highly specific, to be considered as an insoluble particulate bound enzyme, prepared from mitochondria.

form: 4., D-erythro-diol:ketol-oxidoreductase, type: dMt:NAD-oxidoreductase (EC.1.1.1.67)*.

Organism	Demonstrated reaction	References
Candida albicans	dMt—dF, Rt—lRu,	(CHAKRAVORTY *et al.*, 1962)
Candida utilis	dMt—dF, Rt—lRu, dSt—lSo, dAt—dXu,	(ARCUS & EDSON, 1956; BARNETT, 1968b; CHAKRAVORTY *et al.*, 1962; MCCORKINDALE & EDSON, 1954)
Geotrichum candidum	dMt—dF, Rt—lRu,	(WAN & CHANG, 1965)
Pichia delftensis	dMt—dF, dSt—lSo, dAt—dXu,	(BARNETT, 1968b)
Pichia miso	dMt—dF, dAt—dXu, Rt—lRu,	(ONISHI & SAITO, 1962)
Saccharomyces cerevisiae	dMt—dF,	(MÜLLER, 1937; WOLF & KAPLAN, 1956)
Saccharomyces rouxii	dMt—dF, dAt—dXu,	(BLAKELY & SPENCER, 1962; INGRAM & WOOD, 1965)
Torulopsis candida	dMt—dF, dSt—lSo, dAt—dXu,	(BARNETT, 1968b)
Agaricus campestris	dMt—dF,	(EDMUNDOWICZ & WRISTON, 1963)
Aspergillus oryzae	dMt—dF, dSt—lSo, dAt—dXu,	(HORIKOSHI *et al.*, 1965)
Penicillium chrysogenum	dMt—dF,	(CHIANG & KNIGHT, 1959)
Schizophyllum commune	dMt—dF, dSt—lSo,	(NIEDERPRUEM *et al.*, 1965)
Acetobacter suboxydans	dMt—dF, dSt—lSo, Rt—lRu, dAt—dXu,	(ARCUS & EDSON, 1956; FULMER & UNDERKOFLER, 1947; MCCORKINDALE & EDSON, 1954; MOSES & FERRIER, 1962)
Aerobacter aerogenes	dMt—dF, dSt—lSo, dAt—dXu,	(ANDERSON & WOOD, 1962; FOSSITT *et al.*, 1964; FOSSITT & WOOD, 1966; LIN, 1961)

Table 3 (continued)

Lactobacillus brevis	dMt—dF,	(HORECKER, 1966; KATAGIRI *et al.*, 1960)
Pseudomonas fluorescens	dMt—dF, dSt—lSo,	(SEBEK & RANDLES, 1952; SHAW, 1962)
Sorbus aucuparia	dMt—dF, dSt—lSo, Rt—lRu, dAt—dXu,	(MCCORKINDALE & EDSON, 1954)

* dAt:NAD-oxidoreductase (EC.1.1.1.11.) is to be systematized here.

group: b., NADP-enzymes, *type:* dMt:NADP-oxidoreductase.

Geotrichum candidum	dMt—dF,	(CHANG & LI, 1963, 1964)
Saccharomyces acidifaciens	dMt—dF, dSt—lSo, dAt—dXu,	(BARNETT, 1968b)
Torulopsis candida	dMt—dF, dSt—lSo, dAt—dXu,	(BARNETT, 1968b)
Agaricus campestris	dMt—dF,	(DOUDOROFF, 1961)
Aspergillus oryzae	dMt—dF, dSt—lSo, dAt—dXu,	(HORIKOSHI *et al.*, 1965)
Acetobacter suboxydans	dMt—dF, dSt—lSo, dAt—dXu,	(BYGRAVE & SHAW, 1961; CUMMINS *et al.*, 1957a,b; KERSTERS & DE LEY, 1966)
Lactobacillus brevis	dMt—dF,	(HORECKER, 1966)
silkworm egg	dSt—lSo,	(CHINO, 1963)

group: c., Cytochrome-enzymes, *type:* dMt:Fe^{3+}-CC-oxidoreductase (EC. 1.1.1.2.).

Acetobacter suboxydans	dMt—dF, dSt—lSo, dAt—dXu, Rt—lRu,	(ARCUS & EDSON, 1956; EDSON & SHAW, 1966; WOOD *et al.*, 1961)
other organisms (microbe also)	dSt—lSo,	(CHELDELIN, 1960)

class: B., polyol:aldose interconversion.
form: 1. and 2., L-alditol:diol-oxidoreductase, *group:* a., NADP-enzymes, *type:* L-alditol:NADP-oxidoreductase (EC.1.1.1.21.).

Organism	Demonstrated reaction	References
Candida albicans	Xt—dX, lAt—lA, Rt—dR, Gt—dG, dSt—dD,	(HORECKER, 1962; SCHER & HORECKER, 1966; VEIGA *et al.*, 1960)
Candida tropicalis	Xt—dX, lA—lAt—lLy, Rt—dR,	(KARASEVITCH & IPATOVA, 1967, 1968)
Candida utilis	Xt—dX, lAt—lA, Rt—dR, dSt—dD,	(BARNETT, 1968b; HORECKER, 1962; SCHER & HORECKER, 1966; VEIGA *et al.*, 1960)
Geotrichum candidum	Xt—dX, lAt—lA, Rt—dR,	(CHANG *et al.*, 1962; MORET & SPERTI, 1962)
Rhodotorula glutinis	Xt—dX, lAt—lA, Rt—dR, dSt—dD, Gt—dG,	(CHIANG & KNIGHT, 1959, 1960a,b, 1966; CHIANG *et al.*, 1958)
Saccharomyces mellis	Xt—dX,	(WEINBERG, 1962)

Table 3 (continued)

Torulopsis candida	Xt—dX, lAt—lA, Rt—dR, dSt—dD, Gt—dG,	(BARNETT, 1968b)
Aspergillus sp.	Xt—dX, lAt—lA, Rt—dR, dSt—dD, Gt—dG,	
Penicillium chrysogenum	Xt—dX, lAt—lA, Rt—dF, dSt—dD, Gt—dG,	(CHIANG & KNIGHT, 1959, 1960a, 1966; CHIANG *et al.*, 1958)
Schizophyllum commune	Xt—dX, lAt— lA, dSt—dD, Gt—dG,	(NIEDERPRUEM *et al.*, 1965)
mammalian organs	dSt—dD, Gt—dG,	(EDSON, 1961; HERS, 1956, 1960a, b; KINOSHITA *et al.*, 1962)
group: b., FAD-enzymes, *type:* dSt:FAD-oxidoreductase.		
Acetobacter suboxydans	dSt—dD,	(SHOCKLEY & PRIDE, 1959; WIDMER *et al.*, 1956)
group: c., others, *type:* "St-dehydrogenase"		
Acetobacter suboxydans	dSt—hexose, dD—hexitol, dMt—hexose,	(CUMMINS *et al.*, 1957b; WIDMER *et al.*, 1956)

lX is not only poorly transported by the two species but its transformation is lacking in both. The negativity of dSt and Rt utilization in *C. lipolytica* may be caused by a transport defect. At the same time we have no reasonable explanations for the negative utilization results of *C. lipolytica* with dLy and dA (the "−" cases of the "±" result), and for that of *C. pelliculosa* with dA. Both the transport processes and the reactions following the redox ones (demonstrated in this study) do not seem to be responsible for these utilization defects.

2.2.5. General comment. Four reactions, previously not known in yeasts, were demonstrated by us. These are lX (*C. utilis*, *Rh. gracilis*), dA (all the five strains), dLy (all the five strains) and dMn (*C. utilis*, *Pc. tropicalis*) reduction with NADP·H, where on a structural basis the production of Xt, dAt, dAt and dMt seems to be probable. According to Fig. 8, these activities would have been ascribed to the presence of two enzymes. The dMt $\xrightarrow{\text{NADP}}$ dMn reaction was, however, mentioned by some authors (BARNETT, 1968b; CHAKRAVORTY *et al.*, 1962; VEIGA *et al.*, 1960), as a very weak reaction or as result of a "contaminant"?!

It can be stated, however, that not enough is known about the specificity of polyol–sugar interconverting enzymes. The main questions are as follows.

1. Are there individual enzymes with narrower specificity (down to such with one substrate) within the four main groups of NAD-dependent polyol–ketose oxidoreductases?

2. Is the activity of alditol : NADP-oxidoreductase limited to the two configurational groups characterized by the same position of the first hydroxyl?

3. If the answer to question 2 is positive, how many (one or more) enzymes are acting in the reactions of the substrates not being the member of the previous two configurational groups?

4. If more than one enzyme are involved, how can they be systematized or grouped?

It became clear that the correlations of polyol and sugar utilization in yeasts lies in the wider specificity range of oxidoreductases, and also that the exceptions of the correlation rules are due rather to transport and/or intermediary metabolic deficiencies than to the narrow substrate spectrum of the oxidoreductases.

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EFFECT OF GAMMA RADIATION ON SOME EMBRYONIC STAGES OF TWO STORED-DATE INSECT SPECIES

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(Received October 10, 1973)

The saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.), and the fig moth, *Ephestia (Cadra) cautella* (Walker) are good representatives of insects that infest dates and other foodstuffs causing annually a considerable damage.

These two insects differ from each other in many aspects and essentially in that the beetle possesses monocentric chromosomes while the moth has chromosomes with diffuse centromeres. Therefore the mechanism of their response to radiation is expected to be quite different at certain developmental stages where mitotic and/or meiotic divisions are involved.

Different age-groups (with 12-h intervals) of eggs, of both species, irradiated with 10 krad of gamma radiation (from ^{60}Co) showed a differential radiosensitivity which decreased as the egg age increased. The hatchability of irradiated eggs of the two species significantly increased in a direct relation to the age in hours ($P \leq 0.05$). However, *Ephestia* eggs showed a higher radioresistance in the present test than *Oryzaephilus* eggs. This resistance to gamma radiation might be due to the peculiar mechanism of irradiated chromosomes with diffuse centromeres during mitosis.

It is concluded that in order to find the proper recommended dose for disinfestation and sterilization purposes, refined studies on the response of embryonic and other developmental stages of different insect species to radiation should be thoroughly carried out.

Insects that live on stored foods are mainly related to species of *Coleoptera* and *Lepidoptera* (BOLES & MARZKE, 1966). About seven lepidopterous and five coleopterous species infest ripe date fruits in Iraq causing annually a considerable damage (HUSSAIN, 1963). The sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.) (*Coleoptera: Cucujidae*), and the fig moth, *Ephestia (Cadra) cautella* (Walker) (*Lepidoptera: Pyralidae*) are good representatives of insects that feed on dates and other stored agricultural products. These two insects usually infest dates and continue breeding in the stores for several generations as long as dates (or other foodstuffs) are stored. Methyl bromide is the main fumigant being used in stores and packing houses (HUSSAIN, 1963; VINCENT & LINDGREN, 1972). However, the most effective fumigants do not kill all the developmental stages of the insects (HORNE & BROWNELL, 1962; VINCENT & LINDGREN, 1972). Eggs and pupae proved to be somewhat resistant to all currently used fumigants. In addition to that, residue accumulation in the fumigated commodity with methyl bromide and the formation of methyl chloride as a result of methyl bromide deterioration (DENNIS *et al.*, 1972) might pose serious problems limiting the use of this chemical as a fumigant. A more

effective method of control, other than using chemicals, is therefore urgently needed (AHMED *et al.*, 1972). Generally, gamma radiation seems to offer more desirable solutions in killing and sterilizing insects. Before adopting radiation techniques for such purposes, the effectiveness of different doses of radiation against all stages of insect development should be thoroughly assessed.

The two above mentioned species differ from each other in many aspects and essentially in that the beetle possesses monocentric chromosomes (KASTURI BAI & SUGANDI, 1968, and references therein) while the moth has chromosomes with diffuse centromeres (VIRKKI, 1963). Therefore, the mechanism of their response to gamma radiation is expected to be quite different at certain developmental stages where mitotic and/or meiotic divisions are involved. The present studies deal with the effect of 10 krad of gamma radiation on the hatchability of the eggs of both species treated at different intervals after laying.

1. Materials and methods

Eggs of *Oryzaephilus surinamensis* were collected every 12 h from stock cultures maintained in containers with wheat flour (HAFEZ & WAKID, 1967) at 28±2°C and 40—60% RH. *Ephestia cautella* eggs were obtained by confining an equal number of males and females in a lantern globe with a mesh screen put on a petri dish. Eggs laid usually dropped through the screen into the petri dish and were collected every 12 h, too. All eggs used in the present studies were kept in an incubator with 25±1°C and a relative humidity of about 50—70%.

The eggs collected were divided into 6 age-groups from 0 to 72 h old, with 12 h intervals. Different age-groups were treated with 10 krad of gamma radiation (from ^{60}Co) at a dose rate of about 450 krad h^{-1}. Untreated eggs were kept alongside as controls. At least 8 replicates were carried out for each age-group.

2. Results and conclusions

The data presented in Tables 1 and 2 show the per cent hatch of irradiated (as well as unirradiated) *Ephestia* and *Oryzaephilus* eggs, respectively.

A dose of 10 krad of gamma radiation prevented hatching of 0—24 h old eggs in both insects. However, egg hatch commenced in the third age-group (*i. e.* 24—36 h old at the time of irradiation) of *Ephestia* eggs, whereas *Oryzaephilus* eggs treated prior to the fifth age-group (48—60 h) failed to hatch. It is evident that in the older eggs more nuclear divisions took place rendering them somewhat more radioresistant. Consequently, as the age of the egg increased, radioresistance also increased. The 10-krad treated eggs in the older age-groups of both species showed progressively a very highly significant increase

Table 1

Effect of 10 krad of gamma radiation on Ephestia cautella eggs treated at different intervals after laying

Age of eggs when irradiated (hours)	No. of replicates	No. of eggs examined	% egg hatch ±S. D.	*t* values	Probability of error (P)
0—12	12	300	0		
12—24	12	300	0		
24—36	12	300	2.67 ± 3.11		
				6.52	≦0.001
36—48	12	300	23.0 ± 10.25		
				16.38	≦0.001
48—60	12	300	89.33 ± 9.39		
				2.34	≦0.05
60—72	14	350	95.43 ± 3.08		
				2.64	≦0.025
Control (unirradiated)	12	300	84.67 ± 15.73		

Table 2

Effect of 10 krad of gamma radiation on Oryzaephilus surinamensis eggs treated at different intervals after laying

Age of eggs when irradiated (hours)	No. of replicates	No. of eggs examined	% egg hatch ±S.D.	*t* values	Probability of error (P)
0—12	10	250	0		
12—24	10	250	0		
24—36	10	250	0		
36—48	8	200	0		
48—60	8	200	31.0 ± 16.52		
				4.73	≦0.001
60—72	8	200	67.0 ± 13.81		
				0.05	>0.5
Control (unirradiated)	21	445	66.74 ± 15.05		

in hatchability (Tables 1 and 2), where $P \leq 0.001$ in all cases, till the per cent hatch reached a level that showed no significant difference from the unirradiated controls. This level was obtained when *Ephestia* eggs were 48—60 h old (89% hatch) which does not significantly differ from the control ($t = 0.89$ and $P > 0.2$). On the other hand, hatchability of the irradiated *Oryzaephilus* eggs reached the control level only when they were 60—72 h old at the time of exposure ($t = 0.05$, $P > 0.5$), see Table 2.

It is interesting to mention that irradiation of 60—72 h old *Ephestia* eggs resulted in a significant increase in the hatchability as compared with the control, where $t = 2.64$ and $P \leq 0.025$. This observation is not easy to elucidate.

However, the overlapping of stages among different eggs laid at different times within one age-group might have caused some discrepancy in the results, hence interpretation became difficult (Table 1), though a more or less similar effect has been reported concerning eggs laid by mites exposed to the same dose (MELVILLE, 1958).

When age was plotted against the per cent hatch of each species, the data points fell on straight lines (Fig. 1).

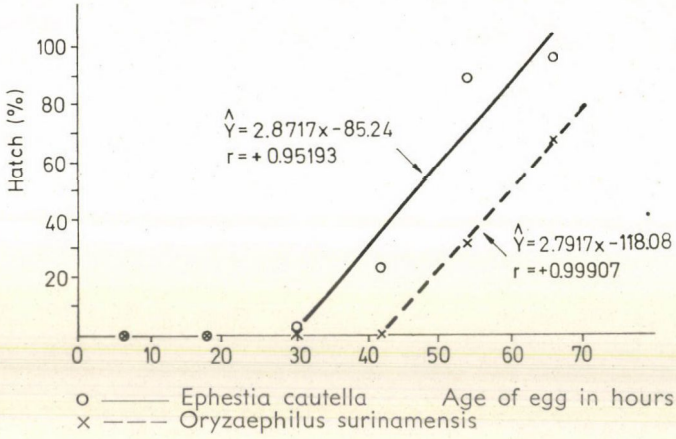

Fig. 1. The relationship of the age and the per cent hatch of irradiated eggs of two stored-date insects

The hatchability of irradiated eggs of the two species significantly increased in a direct relation to the age in hours and regression coefficients in both cases were significantly different ($P \leq 0.05$) from 0. Close correlations ($r = 0.951$ and 0.999 for *Ephestia* and *Oryzaephilus* eggs, respectively) existed between the age and hatchability. As the age of eggs increased by one hour the hatchability of *Ephestia* eggs increased by an average of 2.87 % starting from 30 h old eggs, while *Oryzaephilus* eggs increased by 2.79 % when they were more than 42 h old at the time of exposure to radiation.

These results indicate that the killing effect of 10 krad of gamma radiation on the young eggs before hatching might be ascribed to inhibition of mitosis by this dose which has been administered after one or a few nuclear divisions, while in old eggs, where more mitotic cycles took place, the death occurs predominantly after hatching (VON BORSTEL & AMAND, 1963). Thus the significant increase in the hatchability of the irradiated old eggs does not reflect the deleterious effects of gamma radiation since high mortality in subsequent stages should be expected (EL-SAYED & GRAVES, 1969; BROWN *et al.*, 1972).

Therefore, killing old embryos of both species before hatching requires higher doses, a fact which is also known in other species related to various insect orders (NÖTHEL, 1968).

Fig. 1 shows that *Ephestia* eggs exhibited a higher radioresistance (see also Tables 1 and 2). This resistance to gamma radiation might be due to the peculiar mechanism of irradiated chromosomes with diffuse centromeres (VIRKKI, 1963) during mitosis as the behaviour of chromosomal fragments in respect of spindle fibers is normal (NORTH & HOLT, 1968). At the same time, the acentric fragments caused by irradiation of monocentric chromosomes in the beetle (KASTURI BAI & SUGANDI, 1968) would not be able to proceed further during mitotic division and, therefore, lead to dominant lethality.

The results of these tests clearly demonstrate that in order to find the proper dose for controlling these pests, investigations on the response of various embryonic and other developmental stages to different doses of radiation should be adequately made.

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